

BEST AVAILABLE COPY

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date
5 February 2004 (05.02.2004)

PCT

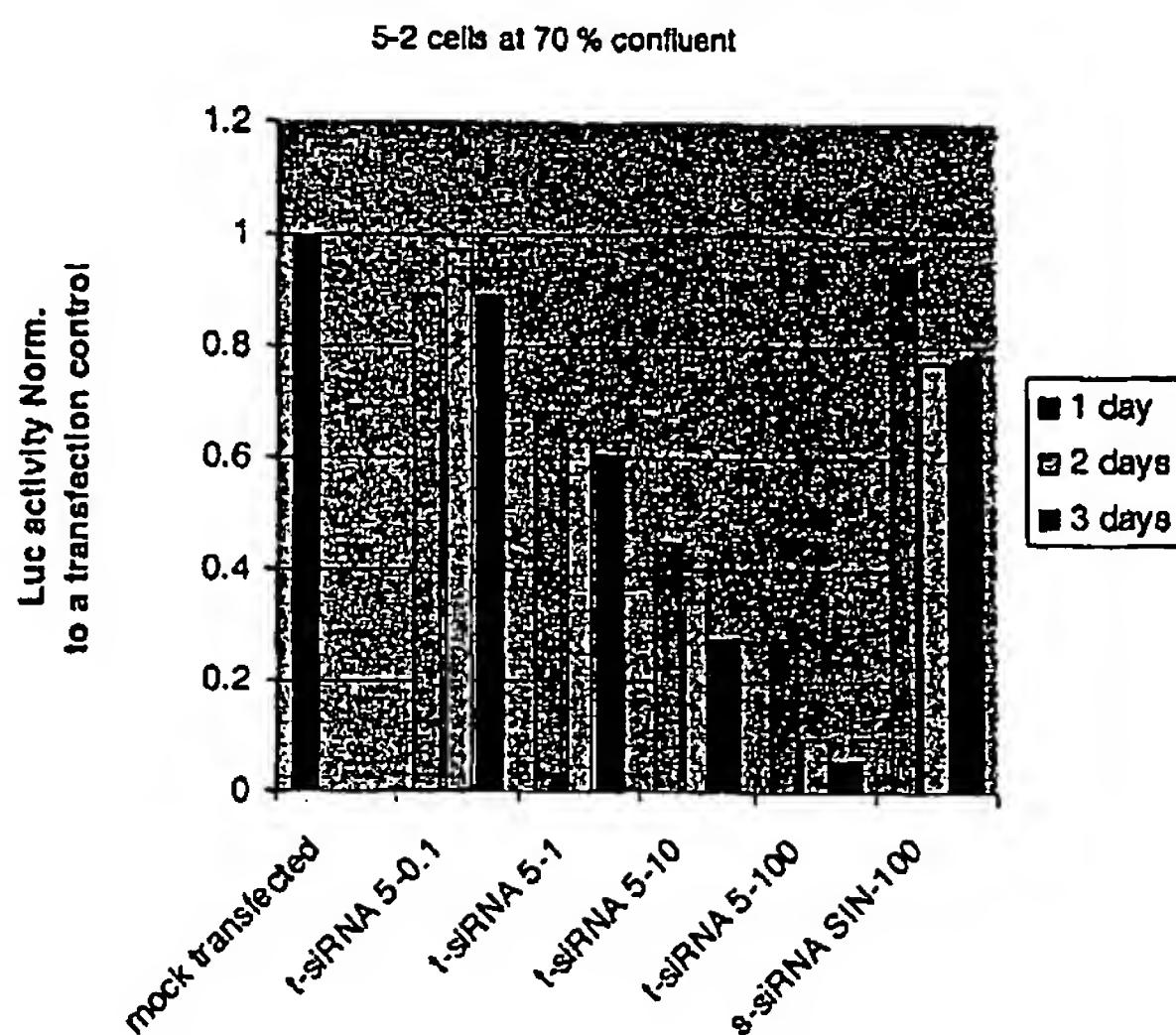
(10) International Publication Number
WO 2004/011647 A1

- (51) International Patent Classification⁷: C12N 15/11, A61K 31/711
- (21) International Application Number: PCT/US2003/023104
- (22) International Filing Date: 25 July 2003 (25.07.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/398,605 26 July 2002 (26.07.2002) US
- (71) Applicant (*for all designated States except US*): CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): HAN, Jang [US/US]; c/o Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 (US). SEO, Mi Young [US/US];
- c/o Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 (US). HOUGHTON, Michael [US/US]; c/o Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 (US).
- (74) Agents: PEET, Richard, C. et al.; Foley & Lardner, Washington Harbour, 3000 K Street, N.W., Suite 500, Washington, D.C. 20007-5143 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,

[Continued on next page]

(54) Title: MODIFIED SMALL INTERFERING RNA MOLECULES AND METHODS OF USE

The Effect of siRNA₅ on HCV Replication In Huh 5-2 Cells



(57) Abstract: The present invention provides double-stranded RNA molecules that mediate RNA interference in target cells, preferably hepatic cells. The invention also provides double-stranded RNA molecules that are modified to be resistant to nuclease degradation, which inactivates a virus, and more specifically, hepatitis C virus (HCV). The invention also provides a method of using these modified RNA molecules to inactivate virus in mammalian cells and method of making modified small interfering RNAs (siRNAs) using human Dicer.

WO 2004/011647 A1



SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

Modified Small Interfering RNA Molecules and Methods of Use

BACKGROUND OF THE INVENTION

The present invention relates to the field of nucleic acid detection and to the phenomenon of RNA silencing, or RNA interference (RNAi). RNA silencing constitutes a phenomenon wherein non-coding RNA molecules mediate specific gene suppression in an organism. In nature, the phenomenon protects an organism's genome from foreign, invading nucleic acids such as transposons, trangenes and viral genes.

The introduction of double-stranded RNA (dsRNA) into a cell triggers RNA silencing, which then degrades endogenous mRNA corresponding to the dsRNA. RNA silencing pathways involve a conversion of dsRNA into short interfering RNAs (siRNAs) that direct ribonucleases to homologous mRNA targets (Baulcombe *et al.*, 2001). An enzyme called Dicer processes the dsRNA into siRNAs, which are 20-25 nucleotides long. The siRNAs then assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). Subsequently, the siRNAs guide the RISCs to complementary RNA molecules, where the RISCs cleave and destroy the target mRNA. Small amounts of dsRNA can silence a large amount of target mRNA due to an amplification component of RNA silencing (Fire *et al.*, *Nature*, 391:806-811 (1998)).

The first evidence that dsRNA produces efficient gene silencing through RNAi came from studies on the nematode *Caenorhabditis elegans* (Fire *et al.*, *Nature*, 391:806-811 (1998) and U.S. Patent No. 6,506,559). Later studies in the fruit fly *Drosophila melanogaster* demonstrated that RNAi is a multi-step mechanism (Elbashir *et al.*, *Genes Dev.*, 15(2): 188-200 (2001)).

Although dsRNA can mediate gene-specific interference in mammalian cells (Wianny, F. and Zernicka-Goetz, M., *Nature Cell Biol.* 2:70-75 (2000) Svoboda, P. *et al.*, *Development* 17:4147-4156 (2000)), the use of RNAi in mammalian somatic cells is often limited by a triggering of dsRNA-dependent protein kinase (PKR), which inactivates the translation factor eIF2a, causes a generalized suppression of protein synthesis and often times causes apoptosis (Gil, J. and Esteban, M., *Apoptosis* 5:107-114(2000)).

Recently, siRNA of approximately 21 or 22 base pairs in length, corresponding to targeted RNA or DNA sequences, were shown to disrupt the expression of the targeted sequences in mammalian cells (Elbashir, S.M., *et al.*, *Nature* 411: 494-498 (2001)). However, it is not clear that all RNA or DNA sequences of a mammalian cell's genome are susceptible to siRNA. It is also uncertain that every mammalian cell type possesses the necessary machinery for effectuating gene-specific suppression using siRNA. Further, siRNA is of limited use for at least two reasons: (a) the transient nature of the suppression effect seen in cells where the siRNA has been administered, and (b) the necessity for chemical synthesis of siRNAs before their use (Tuschl, T., *Nature Biotech.*, 20: 446-448 (2002)). Also, since siRNAs are unstable *in vivo*, their long-term effectiveness is limited.

An invention that addresses these challenges will improve the utility of RNAi for treating human disease at the level of nucleic acid activity. In particular, such an invention will make RNAi a more practical therapy for viral infections, such as infections with HCV. Current therapies for such viral infections are very limited, and tend to have poor response rates.

SUMMARY OF THE INVENTION

The present invention provides double-stranded RNA (dsRNA) molecules that mediate RNA interference in target cells. In particular, it provides small interfering RNAs (siRNAs) that inhibit viral replication in infected cells. Preferred dsRNA molecules of the invention correspond to hepatitis C virus (HCV) nucleic acids, and inhibit replication of HCV in hepatic cells.

In another aspect, the invention provides modified dsRNA, including siRNA, molecules that are protected against nuclease degradation, but are able to inhibit viral replication in mammalian cells.

The invention also provides methods of inhibiting viral replication in infected cells by administering dsRNA or siRNA molecules. Modified dsRNA and siRNA molecules are particularly useful in these methods, as they are nuclease resistant, yet retain the biological activity of being able to inhibit viral replication by targeting an RNA sequence in a virus.

The invention further provides a method of making modified siRNAs that target a viral RNA or DNA sequence. The method comprises preparing a dsRNA fragment that contains at least one modified ribonucleotide in at least one strand, and cleaving the dsRNA fragment with Dicer enzyme, resulting in more than one modified siRNA.

Other objects, features and advantages of the invention will become apparent from the following detailed description. The description and specific examples indicate preferred embodiments, but should not be considered limiting, as various modifications within the scope of the invention will become apparent to those skilled in the art from the detailed description. Further, the examples demonstrate the principle of the invention, but cannot be expected to specifically illustrate all useful applications.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the sequence and secondary structure of the 5' UTR from the HCV genome. It also provides specific sequences of siRNAs for inducing RNAi toward HCV in hepatic cells.

Fig. 2 provides sequences for several HCV-specific siRNAs that are useful for inducing RNAi toward HCV in hepatic cells. Each HCV-specific siRNA is identified by the designation provided in the first column.

Fig. 3 shows the nucleotide sequence of the SARS coronavirus.

Fig. 4 is a schematic representation of the open reading frames of the SARS coronavirus.

Fig. 5 depicts a subgenomic HCV replicon contained in the hepatoma cell line Huh 7, which was used to test the efficacy of siRNA in human liver cells.

Fig. 6 depicts the dose response of normalized luciferase activity in Huh-7 cells containing the subgenomic HCV replicon (5-2 line), that were administered different concentrations of siRNA5. Luciferase activity, which was measured at 1, 2 and 3 days post-transfection, fell with increasing doses of siRNA. The luciferase assay was performed using a Luciferase assay system available from Promega Corp. (Madison, WI), according to the manufacturer's instructions.

Fig. 7 depicts the sequence specificity of siRNA5 for inducing HCV-directed RNAi in Huh-7 liver cells.

Fig. 8 demonstrates that siRNA5 is not toxic to Huh-7 cells. ATPase levels were assayed using an ATPase assay kit available from Promega Corp. (Madison, WI), according to the manufacturer's instructions.

Fig. 9 depicts the effects of siRNA5 on HCV replication in 21-5 cells (Huh-7 cells containing full-length HCV), as measured by RNA assay. RNA levels were assayed using a TaqMan™ RNA kit (F. Hoffman La-Roche, Switzerland), according to the manufacturer's instructions. Values are normalized.

Fig. 10 demonstrates that siRNA5 does not affect the viability of Huh 5-2 cells. Specifically, mRNA encoding GAPDH, an enzyme essential to glycolysis was measured in Huh 5-2 cells transfected with siRNA5 or GAPDH-specific siRNA. The graph demonstrates that siRNA5 did not affect RNA levels of GAPDH. GAPDH was measured using a TaqMan™ RNA kit (F. Hoffman La-Roche, Switzerland), according to the manufacturer's instructions. Values are normalized.

Fig. 11 depicts a dose response of normalized luciferase activity in Huh 7 cells containing a subgenomic HCV replicon (5-2 line) that were administered different concentrations of 2'-fluoro-siRNA (2'-F-GL2), which targets the fruit fly luciferase gene. Luciferase activity, which was measured at 2 days post-transfection, fell with

increasing doses of siRNA. The luciferase assay was performed using a Firefly Luciferase kit (Promega Corp., Madison, WI), according to the manufacturer's instructions.

Fig. 12 demonstrates an inhibition of luciferase activity in 5-2 cells using the siRNA Chol-GL2 in the absence of liposomes.

Fig. 13 depicts an autoradiograph of 5'-labeled siRNA duplexes separated by PAGE, and shows the stability of 2'-fluoro-modified siRNA (2'-F-GL2) incubated in human serum for up to 10 days. The siRNA duplexes were subjected to incubation with human serum and analysis by 20% PAGE. The composition of the lanes is as follows: Lanes 1, 11 and 21: ^{32}P -end labeled siRNA alone; Lanes 2-10, 12-20 and 22-25: siRNA incubated with human serum. Lanes 2 & 12, 1 min ; Lanes 3 & 13, 5 min ; Lanes 4 & 14, 15 min ; Lanes 5 & 15, 30 min ; Lanes 6 & 16, 1 hr ; Lanes 7 & 17, 2 hr ; Lanes 8 & 18, 4 hr ; Lanes 9 & 19, 8 hr ; Lanes 10 & 20, 24 hr ; Lanes 22, 24 hr ; Lanes 23, 48 hr ; Lanes 24, 120 hr ; Lanes 25, 240 hr incubation, respectively.

Fig. 14 demonstrates the use of recombinant human dicer to convert fluorinated dsRNA into 2'F-siRNA. The composition of the lanes is as follows: Lane 1: size marker, λ \HindIII+ ϕ X174\HaeIII; Lane 2: ribo/ribo homoduplex RNA; Lane 3: ribo/2'-F heteroduplex RNA; Lane 4: 2'-F/ribo heteroduplex RNA; Lane 6: size marker, 10bp DNA ladder; Lane 7: ribo/ribo homoduplex siRNA; Lane 8: ribo/2'-F heteroduplex siRNA; Lane 9: 2'-F/ribo heteroduplex siRNA; Lane 10: 2'-F/2'-F homoduplex siRNA.

Fig. 15 shows a dose response of normalized luciferase activity in Huh-7 cells containing the subgenomic HCV replicon (5-2 line) to HCV-specific siRNAs. Luciferase activity fell with increasing doses of each siRNA.

Fig. 16 shows that cholesterol shows a dose response of normalized luciferase activity in Huh-7 cells containing the subgenomic HCV replicon (5-2 line) to cholesterol-modified GL2 siRNA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides dsRNA molecules that are about 10 to about 30 nucleotides long, and that mediate RNA interference in target cells. Preferably, the inventive molecules are chemically modified to confer increased stability against nuclease degradation, but retain the ability to bind to target nucleic acids.

As used herein, "RNA interference" (RNAi) refers to sequence-specific or gene specific suppression of gene expression (protein synthesis) that is mediated by siRNA, without generalized suppression of protein synthesis. While the invention is not limited to a particular theory or mode of action, RNAi may involve degradation of messenger RNA (mRNA) by an RNA-induced silencing complex (RISC), preventing translation of the transcribed mRNA. Alternatively, it may involve methylation of genomic DNA, which shunts transcription of a gene. The suppression of gene expression caused by RNAi may be transient or it may be more stable, even permanent.

"Gene suppression", "targeted suppression", "sequence-specific suppression", "targeted RNAi" and "sequence-specific RNAi" are used interchangeably herein. Furthermore, sequence-specific suppression, as used herein, is determined by separately assaying levels of the protein targeted for suppression in cells containing the siRNA (experimental cells) and in cells not containing the identical siRNA (control cells), then comparing the two values. Experimental and control cells should be derived from the same source and same animal. Also, control and experimental cells used in determining the level or quantity of gene suppression should be assayed under similar, if not identical, conditions.

RNA is a polymer of ribonucleotides, each containing the sugar ribose in association with a phosphate group and a nitrogenous base (typically, adenine, guanine, cytosine, or uracil). Like its cousin, DNA, RNA can form complementary hydrogen bonds. Therefore, RNA may be double-stranded (dsRNA), single-stranded (ssRNA) or double-stranded with a single-stranded overhang. Common types of RNA include messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), short interfering RNA (siRNA), micro RNA (miRNA) and small hairpin

RNA (shRNA), each of which plays a specific role in biological cells. As used herein, the term "RNA" includes all of these.

"Small interfering RNA" (siRNA) refers to double-stranded RNA molecules from about 10 to about 30 nucleotides long that are named for their ability to specifically interfere with protein expression. Preferably, siRNA molecules are 12-28 nucleotides long, more preferably 15-25 nucleotides long, still more preferably 19-23 nucleotides long and most preferably 21-23 nucleotides long. Therefore, preferred siRNA molecules are 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 28 or 29 nucleotides in length.

The length of one strand designates the length of an siRNA molecule. For instance, an siRNA that is described as 21 ribonucleotides long (a 21-mer) could comprise two opposite strands of RNA that anneal together for 19 contiguous base pairings. The two remaining ribonucleotides on each strand would form an "overhang." When an siRNA contains two strands of different lengths, the longer of the strands designates the length of the siRNA. For instance, a dsRNA containing one strand that is 21 nucleotides long and a second strand that is 20 nucleotides long, constitutes a 21-mer.

siRNAs that comprise an overhang are desirable. The overhang may be at the 5' or the 3' end of a strand. Preferably, it is at the 3' end of the RNA strand. The length of an overhang may vary, but preferably is about 1 to about 5 bases, and more preferably is about 2 nucleotides long. Preferably, the siRNA of the present invention will comprise a 3' overhang of about 2 to 4 bases. More preferably, the 3' overhang is 2 ribonucleotides long. Even more preferably, the 2 ribonucleotides comprising the 3' overhang are uridine (U).

siRNAs of the present invention are designed to interact with a target ribonucleotide sequence, meaning they complement a target sequence sufficiently to bind to the target sequence. Preferably the target ribonucleotide sequence derives from a disease producing agent or pathogen. More preferably, the target ribonucleotide sequence is in a virus genome of an RNA virus or a DNA virus. Even more preferably, the virus is selected from the group consisting of hepatitis C virus

(HCV), hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus, Ebola virus, influenza virus, rotavirus, reovirus, retrovirus, poliovirus, human papilloma virus (HPV), metapneumovirus and coronavirus.

Hepatitis C virus (HCV) is a highly preferred virus target. Figure 1 and Figure 2 disclose the nucleic acid sequences for several HCV-specific siRNA molecules. Among those shown, siRNA5, siRNAC1, siRNAC2, siRNA5B1, siRNA5B2, and siRNA5B4 have shown particularly good activity, and therefore are highly preferred. siRNAs at least 80%, 90%, or 95%, identical to these highly preferred siRNAs also constitute part of the invention..

Another preferred virus target is the coronavirus, which is associated with upper respiratory infections in humans and recently has been linked with SARS (severe acute respiratory syndrome). Coronavirus has the largest known RNA virus genome, 32 kilobases long, and its genome is composed of positively stranded RNA. (See Figure 5) Each coronavirus mRNA has a 5'-end leader sequence of 60 to 80 nucleotides that is identical to the 5'-UTR of genomic RNA approximately 200 nucleotides long. (See Figure 6) These sequences are highly conserved, and therefore, provide an excellent source of target sequences for which siRNAs. See Fundamental Virology, 3rd Ed., Chapter 18, p. 541-560 (Eds. Fields, Knipe and Howley), Lippincott-Raven (1995). In one embodiment, the entire leader sequence (nucleotides 1-72) is targeted. In another embodiment, one or more sections of the leader sequence is targeted. In a preferred embodiment, nucleotides 64-72 (TAAACGAAC) of the leader sequence are targeted. siRNA targeted to the coronavirus may be modified or unmodified.

In one embodiment, the invention provides an siRNA molecule comprising a ribonucleotide sequence at least 80% identical to a ribonucleotide sequence from a target agent or virus. Preferably, the siRNA molecule is at least 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the ribonucleotide sequence of the target agent or virus. The target can be the entire viral genome, a primary transcript, an open reading frame, or any portion of these. Most preferably, an siRNA will be 100% identical to the nucleotide sequence of a target agent or virus. However, siRNA molecules with insertions, deletions or single point mutations relative to a target may

also be effective. Tools to assist siRNA design are readily available to the public. For example, a computer-based siRNA design tool is available on the internet at www.dharmacon.com.

By way of example, a polynucleotide having a nucleotide sequence at least 95% "identical" to a reference nucleotide sequence means that the polynucleotide's sequence may include up to five point mutations per 100 nucleotides of the reference nucleotide sequence, or 1 point mutation per 20 nucleotides. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the ribonucleotide sequence of a target agent or virus can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, Madison, WI). Bestfit uses the local homology algorithm of Smith and Waterman (*Advances in Applied Mathematics* 2:482-489 (1981)) to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference ribonucleotide sequence and that gaps in homology of up to 5% of the total number of ribonucleotides in the reference sequence are allowed.

The present invention also includes siRNA molecules that have been chemically modified to confer increased stability against nuclease degradation, but retain the ability to bind to target nucleic acids that may be present in cells. In the

case where a target RNA is virus-specific, the modified siRNAs are able to bind to the virus specific RNAs or DNAs, thereby inactivating the virus.

A modified siRNA of the present invention comprises a modified ribonucleotide, and is resistant to enzymatic degradation, such as RNase degradation, yet retains the ability to inhibit viral replication in a cell containing the specific viral target RNA or DNA sequences. The siRNA may be modified at any position of the molecule so long as the modified siRNA binds to a target sequence and is resistant to enzymatic degradation. Modifications in the siRNA may be in the nucleotide base, *i.e.*, the purine or the pyrimidine, the ribose or the phosphate. Preferably, the modification occurs at the 2' position of at least one ribose in an siRNA.

More specifically, the siRNA is modified in at least one pyrimidine, at least one purine or a combination thereof. However, generally all pyrimidines (cytosine or uracil), or all purines (adenosine or guanine) or a combination of all pyrimidines and all purines of the siRNA are modified. More preferably, the pyrimidines are modified, and these pyrimidines are cytosine, a derivative of cytosine, uracil, a derivative of uracil or a combination thereof. Ribonucleotides on either one or both strands of the siRNA may be modified.

Ribonucleotides containing pyrimidine bases found in RNA (cytidine and uridine) can be chemically modified by adding any molecule that inhibits RNA degradation or breakdown of the base, the ribose or the phosphates. As previously noted, the 2' position of ribose is a preferred site for modification. 2' modified siRNAs have a longer serum half-life and are resistant to degradation, relative to unmodified siRNAs or single-stranded RNAs, such as antisense or ribozyme. 2'-modified pyrimidine ribonucleotides can be formed by a number of different methods known in the art.

A preferable chemical modification is the addition of a molecule from the halide chemical group to a ribonucleotide of siRNA. Within the halides, fluorine is a preferred molecule. Besides fluoro-, other chemical moieties such as methyl-, methoxyethyl- and propyl- may be added as modifications. The most preferred

modification, though, is fluoro-modification, such as a 2'-fluoro-modification or a 2',2'-fluoro-modification.

Thus, in a preferred embodiment of the invention, siRNA is modified by the addition of a fluorine molecule to the 2' carbon of a pyrimidine ribonucleotide. The siRNA may be fluorinated completely or partially. For example, only the cytosine ribonucleotides may be fluorinated. Alternatively, only the uracil ribonucleotides may be fluorinated. In a preferred embodiment, both uracil and cytosine are fluorinated. Only one strand, either sense or antisense, of siRNA may be fluorinated. Even partial 2' fluorination of siRNA gives protection against nucleolytic degradation. Importantly, 2' fluorinated siRNA is not toxic to cells, an unexpected result given that fluorine chemistry usually is toxic to living organisms.

In addition, modified siRNAs of the present invention may contain chemical modifications that inhibit viral RNA polymerases. For example, siRNAs may comprise one or more nucleosides that inhibit viral RNA-dependent RNA polymerases. Examples of such nucleosides and other chemical modifications exist in WO 02/057425, WO 02/057287, WO 02/18404, WO 02/100415, WO 02/32920, WO 01/90121, US patent No. 6,063,628 and US published application No. 2002/0019363.

siRNA can be prepared in a number of ways, such as by chemical synthesis, T7 polymerase transcription, or by treating long double stranded RNA (dsRNA) prepared by one of the two previous methods with Dicer enzyme. Dicer enzyme creates mixed populations of dsRNA from about 21 to about 23 base pairs in length from dsRNA that is about 500 base pairs to about 1000 base pairs in size. Unexpectedly, Dicer can effectively cleave modified strands of dsRNA, such as 2' fluoro-modified dsRNA. Before development of this method, it was previously thought that Dicer would not be able to cleave modified siRNA. The Dicer method of preparing siRNAs can be performed using a Dicer siRNA Generation Kit available from Gene Therapy Systems (San Diego, CA).

The invention particularly includes a method of making a modified siRNA that targets a nucleic acid sequence in a virus, comprising (a) preparing a modified-double stranded RNA (dsRNA) fragment containing at least one modified

ribonucleotide in at least one strand, and (b) cleaving the modified-dsRNA fragments with recombinant human Dicer, resulting in more than one modified siRNA. The method may further comprise (c) isolating the modified siRNAs.

In the methods for making siRNA, a dsRNA fragment can be prepared by chemical synthesis or in vitro translation. In one embodiment, the modified siRNA is a 2' modified siRNA in which the modification is at the 2' position of at least one ribonucleotide of said siRNA. The modification is selected from the group consisting of fluoro-, methyl-, methoxyethyl and propyl-modification. Preferably the fluoro-modification is a 2'-fluoro-modification or a 2',2'-fluoro-modification. The pyrimidines, the purines or a combination thereof of the siRNA are modified. More preferably, the pyrimidines are modified, such as cytosine, a derivative of cytosine, uracil, a derivative of uracil or a combination thereof. One or both strands of the siRNA may contain one or more modified ribonucleotide.

The invention further provides a method of inactivating a target agent or virus in a patient by administering to the patient a dsRNA in an effective amount to inactivate the targeted agent or virus. Preferably the dsRNA is modified as described above. RNA interference toward a targeted DNA segment in a cell can be achieved by administering a double-stranded RNA molecule to the cells, wherein the ribonucleotide sequence of the double-stranded RNA molecule corresponds to the ribonucleotide sequence of the targeted DNA segment. Preferably, the dsRNA used to induce targeted RNAi is siRNA.

As used herein "targeted DNA segment" is used to mean a DNA sequence encoding, in whole or in part, an mRNA for a targeted protein, including introns or exons, where suppression is desired. DNA segment can also mean a DNA sequence that normally regulates expression of the targeted protein, including but not limited to the promoter of the targeted protein. Furthermore, the DNA segment may or may not be a part of the cell's genome or it may be extrachromosomal, such as plasmid DNA.

The present invention is particularly directed to a method of inactivating a virus in a patient by administering to the patient an siRNA, preferably a modified siRNA, in an effective amount to inactivate the virus. The siRNA is preferably about

10 to about 30 ribonucleotides in length, more preferably 12-28 ribonucleotides, more preferably 15-25 ribonucleotides, even more preferably 19 –23 ribonucleotides and most preferably 21-23 ribonucleotides.

Also, the method of inactivating a virus preferably utilizes an siRNA that is modified at the 2' position of at least one ribonucleotide of said siRNA. The siRNA may be modified with chemical groups selected from the group consisting of fluoro-, methyl-, methoxyethyl- and propyl-. Fluoro-modification is most preferred, and either a 2'-fluoro-modification or a 2',2'-fluoro-modification is useful in the method. The modification may be at a pyrimidine, a purine or a combination thereof of the siRNA. More preferably the pyrimidines are modified, such as cytosine, a derivative of cytosine, uracil, a derivative of uracil or a combination thereof. In one embodiment, one strand of the siRNA contains at least one modified ribonucleotide, while in another embodiment, both strands of the siRNA contain at least one modified ribonucleotide.

siRNAs useful in treatment methods may also be modified by the attachment of at least one, but preferably more than one, receptor-binding ligand(s) to the siRNA. Such ligands are useful to direct delivery of siRNA to a target virus in a body system, organ, tissue or cells of a patient, such as the liver, gastrointestinal tract, respiratory tract, the cervix or the skin.

In preferred embodiments, receptor-binding ligands are attached to either a 5'-end or a 3'-end of an siRNA molecule. Receptor-binding ligands may be attached to one or more siRNA ends, including any combination of 5'- and 3'-ends. Thus, when receptor binding ligands are attached only to the ends of an siRNA molecule, anywhere between 1 and 4 such ligands may be attached.

The selection of an appropriate ligand for targeting siRNAs to viruses in particular body systems, organs, tissues or cells is considered to be within the ordinary skill of the art. For example, to target an siRNA to hepatocytes, cholesterol may be attached at one or more ends, including any combination of 5'- and 3'-ends, of an siRNA molecule. The resultant cholesterol-siRNA is delivered to hepatocytes in the liver, thereby providing a means to deliver siRNAs to this targeted location. Other

ligands useful for targeting siRNAs to the liver include HBV surface antigen and low-density lipoprotein (LDL).

As another example, siRNA molecules that target Human Immunodeficiency virus type 1 (HIV-1) can be delivered to T lymphocytes where the target nucleic acids are located (Song, E. *et al.*, *J. of Virology*, 77(13): 7174-7181 (2003)). This delivery can be accomplished by attaching, at the 3' -end or 5' -end of siRNA molecules, HIV-1 surface antigen capable of binding to the CD4 surface protein located on T-cells (Kilby, M. *et al.*, *New England J. of Medicine*, 348(22): 2228-38 (2003)).

Similarly, siRNA molecules that target Influenza A virus can be delivered to epithelial cells of the respiratory tract where the target nucleic acids are located (Ge, Q. *et al.*, *Proc. Natl. Acad. of Sciences*, 100(5): 2718-2723 (2002)). This delivery can be accomplished by attaching, at the 3' -end or 5' -end of siRNA molecules, the Influenza virus surface antigen, which is capable of binding to the sialic acid residues located on the surface of the epithelial cells (Ohuchi, M., *et al.*, *J. of Virology*, 76(24): 12405-12413 (2002); Glick, G. *et al.*, *J. of Biol. Chem.*, 266 (35): 23660-23669 (1991)).

Also, siRNA molecules that target respiratory syncitial virus (RSV) can be delivered to epithelial cells of the respiratory tract where the target nucleic acids are located (Bitko, V. *et al.*, *BMC Microbiology*, 1:34 (2001)). This delivery can be accomplished by attaching, at the 3' -end or 5' -end of siRNA molecules, RSV surface antigen (Malhotra, R. *et al.*, *Microbes and Infection*, 5: 123-133 (2003)).

As still another example, siRNAs that target Human Papillomavirus (HPV) can be delivered to basal epithelial cells where the target nucleic acids are located (Hall, A. *et al.*, *J. of Virology*, 77(10): 6066-6069 (2003)). This delivery can be accomplished by attaching, at the 3' -end or 5' -end of siRNA molecules, HPV surface antigen capable of binding to heparin sulfate proteoglycans located on the surface of basal epithelial cells (Bousarghin L. *et al.*, *J. of Virology*, 77(6): 3846-3850 (2002)).

Further, siRNAs that target Poliovirus (PV) can be delivered to cells of the nervous system where the target nucleic acids are located (Gitlin, L. *et al.*, *Nature*,

418: 430-434 (2002)). This delivery can be accomplished by attaching, at the 3' -end or 5' -end of siRNA molecules, PV surface antigen capable of binding to the CD155 receptor located on the surface of neurons (He, Y. *et al.*, *Proc. Natl. Acad. of Sciences*, 97 (1): 79-84 (2000)).

As noted, the methods of treatment are intended to target disease-causing agents or pathogens, and more particularly viruses, which can be either RNA viruses or DNA viruses. Preferred viruses are selected from the group consisting of hepatitis C virus (HCV), hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus, Ebola virus, influenza virus, rotavirus, reovirus, retrovirus, poliovirus, human papilloma virus (HPV), metapneumovirus and coronavirus. More preferably the target virus is hepatitis C virus or a coronavirus.

In one aspect, the method utilizes an siRNA prepared by (a) identifying a target ribonucleotide sequence in a virus genome for designing a small interfering RNA (siRNA) and (b) producing a siRNA that has been modified to contain at least one modified ribonucleotide. Preferably, the siRNA comprises a double-stranded RNA molecule with a first strand ribonucleotide sequence corresponding to a ribonucleotide sequence corresponding to a target ribonucleotide sequence in the virus, and a second strand comprising a ribonucleotide sequence complementary to the target ribonucleotide sequence. The first and second strands should be separate complementary strands that hybridize to each other to form a double-stranded RNA molecule. Moreover, one or both of the strands should comprise at least one modified ribonucleotide.

In preferred embodiments of the invention, the siRNA targets a ribonucleotide sequence in the hepatitis C virus genome. The target ribonucleotide sequence comprises a conserved ribonucleotide sequence necessary for HCV replication, and the conserved ribonucleotide sequence is selected from the group consisting of 5'-untranslated region (5'-UTR), 3'-untranslated region (3'-UTR), core, and NS3 helicase. Highly preferred siRNA molecules comprise a sequence at least 80% identical to those of siRNA5, siRNAC1, siRNAC2, siRNA5B1, siRNA5B2, or siRNA5B4. The siRNAs may be unmodified, or modified as described above.

Methods of inhibiting the replication of HCV in cells positive for HCV should not be toxic to the cells, or cause apoptosis in the treated cells. Preferably, the inhibition of HCV replication is specifically tailored to affect only HCV replication in the cells, such that normal growth, division or metabolism is not affected. Cells in which HCV has been shown to replicate include, but are not limited to hepatic cells, B cell lymphocytes and T cell lymphocytes. Preferably, a method of inhibiting the replication of HCV is performed in hepatic cells.

According to the invention, "hepatic cells" can be from any animal source. Further, the hepatic cells may be in cell culture, or part of a tissue, or an organ, in part or in whole. The phrase hepatic cells is meant to include any cell constituting a normal, abnormal or diseased liver cell. Examples of hepatic cells include, but are not limited to, Kupffer cells, hepatocytes and cells comprising a hepatocellular carcinoma. "Hepatic cells" is not meant to include cells that make up discrete structures within the liver, such as endothelial cells lining blood vessels. A tissue or organ containing the hepatic cells may be within a subject or may be biopsied or removed from the animal. Additionally, the tissue may be "fresh" in that the tissue would be recently removed from a subject, without any preservation steps between the excision and the methods of the current invention. Prior to application of the methods of the current invention, the tissue may also have been preserved by such standard tissue preparation techniques including, but not limited to, freezing, quick freezing, paraffin embedding and tissue fixation. Furthermore, the tissue may also be a xenograft or a syngraft on or in another host animal. As used herein, the terms animal and subject are used interchangeably.

According to the invention, "hepatitis C virus," or "HCV," takes its ordinary meaning in the art as of the date of invention. The hepatitis C virus is an RNA virus of the *Flaviviridae* family. For example as used herein, HCV includes, but is not limited to genotypes 1-11 (using the most common genotyping system), with these genotypes being broken down into sub-types, some of which include but are not limited to 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4a, 4b, 4c, 4d, 4e, 5a, 6a, 7a, 7b, 8a, 8b, 9a, 10a and 11a. Further, isolates from individuals consist of closely related yet heterogeneous populations of viral genomes, sometimes referred to as quasispecies.

Pestivirus is yet another target of the present invention. As used herein, "pestivirus" takes its ordinary meaning in the art as of the date of invention. The pestivirus belongs to the family *Flaviviridae*. Pestivirus is widespread throughout the Australian cattle population. It is believed that about 70% of herds are actively infected with pestivirus. Infection of susceptible animals can cause a variety of diseases - some not apparent until well after the initial spread of the virus into a herd. Pestivirus is a genus of viruses that includes hog cholera virus, bovine viral diarrhea virus (BVDV) and border disease virus (BDV) or hairy-shaker disease virus.

siRNA may be administered to a patient by intravenous injection, subcutaneous injection, oral delivery, liposome delivery or intranasal delivery. The siRNA may then accumulate in a target body system, organ, tissue or cell type of the patient.

The present invention also provides a method of inhibiting the replication of a virus in mammalian cells, comprising transfecting cells harboring the virus with a vector that directs the expression of virus-specific siRNA. In one embodiment, the invention provides a method of inhibiting the replication of hepatitis C virus (HCV) in cells positive for HCV, comprising transfecting HCV-positive cells with a vector that directs the expression of an HCV-specific siRNA. The cells may be evaluated to determine if a marker in the cells has been inhibited by the siRNA.

Thus, the invention also provides vectors and host cells comprising a nucleic acid segment encoding the described siRNAs.

Vectors of the present invention may be employed for producing siRNAs by recombinant techniques. Thus, for example, a DNA segment encoding an siRNA may be included in any one of a variety of expression vectors for expressing any DNA sequence. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in a desired host.

The appropriate DNA segment may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA segment in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct siRNA synthesis. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous Sarcoma Virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter. Preferably the promoters of the present invention are from the type III class of RNA polymerase III promoters. More preferably, the promoters are selected from the group consisting of the U6 and H1 promoters. The U6 and H1 promoters are both members of the type III class of RNA polymerase III promoters. The promoters of the present invention may also be inducible, in that expression may be turned "on" or "off." For example, a tetracycline-regulatable system employing the U6 promoter may be used to control the production of siRNA. The expression vector may or may not contain a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader

sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

In one embodiment, the invention provides a vector, wherein the DNA segment encoding the sense strand of the RNA polynucleotide is operably linked to a first promoter and where the DNA segment encoding the antisense (opposite) strand of the RNA polynucleotide molecule of is operably linked to a second promoter. In other words, each strand of the RNA polynucleotide is independently expressed. Furthermore, the promoter driving expression of each strand can be identical or each one may be different from the other promoter.

In another embodiment, the vector of the current invention may comprise opposing promoters. For example, the vector may comprise two U6 promoters on either side of the DNA segment encoding the sense strand of the RNA polynucleotide and placed in opposing orientations, with or without a transcription terminator placed between the two opposing promoters. The U6 opposing promoter construct is similar to the T7 opposing promoter construct as described in Wang, Z. *et al.*, J. Biol. Chem. 275: 40174-40179 (2000). See Miyagishi, M. and Taira, K., Nature Biotech. 20: 497-500 (2002).

In another embodiment, the DNA segments encoding both strands of the RNA polynucleotide are under the control of a single promoter. In one embodiment, the DNA segments encoding each strand are arranged on the vector with a “loop” region interspersed between the two DNA segments, where transcription of the DNA segments and loop region creates one RNA transcript. The single transcript will, in turn, anneal to itself creating a “hairpin” RNA structure capable of inducing RNAi. The “loop” of the hairpin structure is preferably from about 4 to about 6 nucleotides in length. More preferably, the loop is 4 nucleotides in length.

The vector containing the appropriate DNA sequence as described herein, as well as an appropriate promoter or control sequence, may be employed to transform

an appropriate host to permit the host to express the siRNA. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y. (1989), the disclosure of which is hereby incorporated by reference.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, cloning vectors or expression vectors. The vectors may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells may be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. A host cell may be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell may be a prokaryotic cell, such as a bacterial cell. Preferably, host cells are mammalian cells. More preferably, host cells are hepatic cells. Introduction of a construct into host cells can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., et al., Basic Methods in Molecular Biology (1986)).

The term patient, as used herein, refers to an animal, preferably a mammal. More preferably the patient can be a primate, including non-human and humans. The terms subject and patient are used interchangeably herein.

The treatments envisioned by the current invention can be used for subjects with a pre-existing viral infection, or for subjects pre-disposed to an infection. Additionally, the methods of the current invention can be used to correct or compensate for cellular or physiological abnormalities involved in conferring susceptibility to viral infections in patients, and/or to alleviate symptoms of a viral infections in patients, or as a preventative measure in patients.

The method of treating a patient having a viral infection involves administration of compositions to the subjects. As used herein, composition can mean a pure compound, agent or substance or a mixture of two or more compounds, agents or substances. As used herein, the term agent, substance or compound is intended to mean a protein, nucleic acid, carbohydrate, lipid, polymer or a small molecule, such as a drug.

In one embodiment of the current invention, the composition administered to the subject is a pharmaceutical composition. Further, the pharmaceutical composition can be administered orally, nasally, parenterally, intrasystemically, intraperitoneally, topically (as by drops or transdermal patch), buccally, or as an oral or nasal spray. Intranasal delivery of a virus that causes upper respiratory diseases, such as the coronavirus or the metapneumovirus, would be a particularly advantageous delivery mode. The term "parenteral," as used herein, refers to modes of administration that include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. The pharmaceutical compositions as contemplated by the current invention may also include a pharmaceutically acceptable carrier.

"Pharmaceutically acceptable carrier" includes, but is not limited to, a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type, such as liposomes.

A pharmaceutical composition of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

The compositions of the present invention can also contain adjuvants such as, but not limited to, preservatives, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms can be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorb acid, and the like. It can also be desirable to include isotonic agents such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, to prolong the effect of the drugs, it is desirable to slow the absorption from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, can depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium just prior to use.

Solid dosage forms for oral administration include, but are not limited to, capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compounds are mixed with at least one item pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders

such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, acetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form can also comprise buffering agents.

Solid compositions of a similar type can also be employed as fillers in soft and hard filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They can optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms can contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut, corn, germ, olive,

castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Suspensions, in addition to the active compounds, can contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth, and mixtures thereof.

Alternatively, the composition can be pressurized and contain a compressed gas, such as nitrogen or a liquefied gas propellant. The liquefied propellant medium and indeed the total composition is preferably such that the active ingredients do not dissolve therein to any substantial extent. The pressurized composition can also contain a surface active agent. The surface active agent can be a liquid or solid non-ionic surface active agent or can be a solid anionic surface active agent. It is preferred to use the solid anionic surface active agent in the form of a sodium salt.

The compositions of the present invention can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to the compounds of the invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art (see, for example, Prescott, Ed., *Meth. Cell Biol.* 14:33 *et seq* (1976)).

One of ordinary skill in the art will appreciate that effective amounts of the agents of the invention can be determined empirically and can be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt, ester or prodrug form. A "therapeutically effective" amount of the inventive compositions can be

determined by prevention or amelioration of adverse conditions or symptoms of diseases, injuries or disorders being treated. The agents can be administered to a subject, in need of treatment of viral infection, as pharmaceutical compositions in combination with one or more pharmaceutically acceptable excipients. It will be understood that, when administered to a human patient, the total daily usage of the agents or composition of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors: the type and degree of the cellular or physiological response to be achieved; activity of the specific agent or composition employed; the specific agents or composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the agent; the duration of the treatment; drugs used in combination or coincidental with the specific agent; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the agents at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosages until the desired effect is achieved.

Dosing also can be arranged in a patient specific manner to provide a predetermined concentration of the agents in the blood, as determined by techniques accepted and routine in the art. Thus patient dosaging can be adjusted to achieve regular on-going blood levels, as measured by HPLC, on the order of from 50 to 1000 ng/ml.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein can be made without departing from the scope of the invention or any embodiment thereof.

EXAMPLES

The examples demonstrate that siRNA, including modified siRNA, can effectively inhibit viral replication in mammalian cells. Moreover, the examples

show that the inventive siRNAs promote HCV RNA degradation in human liver cells and establish that hepatocytes possess the necessary functional components of modified siRNA-induced silencing. The examples also demonstrate that siRNA technology can be used as a therapy to inhibit HCV replication in host cells. The inventors, by submitting the following examples, do not intend to limit the scope of the claimed invention.

EXAMPLE 1

To test whether siRNA directed to the HCV genome confers intracellular immunity against this human pathogen, a recently developed HCV cell culture systems in human hepatoma cell line, Huh-7, was used. One of the cell lines, 5-2, harbors autonomously replicating subgenomic HCV RNA (Bartenschlager, J. Virol, 2001). The subgenomic replicon carries firefly luciferase gene, allowing a reporter function assay as a measure of HCV RNA replication (Fig. 5). Owing to cell culture adaptive mutations introduced into the genome (Bart), these 5-2 cells replicate HCV RNA at levels of up to 5×10^4 virus particles/cell.

Using T7 transcription, several 21-bp siRNA duplexes against different regions of the 5'-UTR of the HCV genome were made (Fig 5). Briefly, 2 oligo double-stranded DNA molecules comprising the T7 promoter and the 5' UTR of HCV being oriented in either the sense direction or the antisense direction were generated. Each oligo DNA was then transcribed *in vitro* to produce (+) and (-) RNA and then treated with DNAase I to remove the DNA template. The two RNA strands were allowed to anneal at 37°C overnight, generating dsRNA. After treating the dsRNA with RNAase T1 to remove unreacted ssRNA species, the dsRNA was purified for transfection.

Several other siRNA duplexes were designed, including GL2 and GL3, that were directed against the fruit fly and sea pansy luciferase genes, respectively. Using standard transfection techniques, the siRNAs were transfected into the 5-2 cells and luciferase activity was measured to determine the effect of the siRNAs on HCV replication. Luciferase activity was measured 48 hours after transfection. In cells

where siRNA5 was transfected, there was reduced luciferase activity of up to 85%, in a dose responsive manner (Fig. 6). The inhibition of luciferase activity was not seen in cells that were transfected with irrelevant siRNA (SIN). The sequence of SIN was taken from sindbis virus transcription promoter (Fig. 1).

EXAMPLE 2

The sequence specificity of the siRNA5 response was further tested using additional siRNA duplexes, GL2 and GL3. Figure 1 shows that GL2 and GL3 differ from each other by 3-nucleotides. Luciferase activity was reduced by 90% in cells transfected with siRNA5 or GL2, but no significant reduction was seen in cells transfected with GL3 (Fig. 7). The luciferase assay was performed using a Luciferase assay system available from Promega Corp. (Madison, WI), according to the manufacturer's instructions.

EXAMPLE 3

Whether or not siRNA5 was toxic to transfected cells also was tested. Toxicity was measured using an ATPase activity assay. Figure 8 shows that the siRNA5-induced reduction in HCV replication, as seen in Figure 6, was not due to cellular toxicity which is attributed to non sequence-specific RNAi. ATPase levels were assayed using an ATPase assay kit from Promega (Madison, WI) according to the manufacturer's instructions.

EXAMPLE 4

The full-length HCV replicon may possess the ability to adapt and suppress RNAi, thus replicating in spite of the presence of siRNA, as documented in Li, H, Science 296:1319-1321 (2002). To determine the effects of siRNA5 on replication of full-length HCV RNA in Huh-7 cells, from the 21-5 cell line, harboring the selectable full-length HCV replicon, were treated with siRNA5. Levels of HCV RNA were measured by quantitative PCR using TaqMan™ (F. Hoffman La-Roche, Switzerland). The results as seen in Figure 9 show that siRNA-directed silencing reduced steady-

state viral RNA production, even in the setting of an adapted HCV mutant, where RNA replication was very high. Results from both subgenomic and full-length HCV replicons suggest that none of the HCV proteins can suppress RNA interference.

EXAMPLE 5

Whether or not siRNA5 was toxic to transfected cells also was tested. Specifically, mRNA encoding GAPDH, an enzyme essential in glycolysis, was measured in Huh 5-2 cells transfected with siRNA5, or siRNA specific towards the GAPDH sequence. Figure 10 demonstrates that siRNA5 did not affect RNA levels of GAPDH. GAPDH was measured using a TaqMan™ RNA kit (F. Hoffman La-Roche, Switzerland) according to the manufacturer's instructions.

EXAMPLE 6

To test the effectiveness of siRNA5 on inhibiting the ability of HCV to replicate in an infected liver, potions of HCV-infected human liver are xenografted onto transgenic severe combined immunodeficient (SCID) mice according to methods well known to the skilled artisan.

Briefly, once the HCV-infected liver has supplanted the mouse liver, liposome-encapsulated siRNA5, or control liposomes are administered by intravenous injection to the mice through the tail vein, or another accessible vein. The mice are dosed one time a day for 3-10 days.

At the end of the dosing regimen the mice are sacrificed and blood collected and the livers removed. The liver is divided into portions such that a portion is frozen using liquid nitrogen, a portion is fixed for paraffin embedding, and a portion is fixed for sectioning onto slides.

Using the appropriate allotment, HCV RNA is quantified using the TaqMan™ RNA assay kit previously utilized herein to determine the levels of HCV RNA in the liver cells. Further, anti-HCV antibody titers can be measured in the collected blood samples, along with serum ALT levels.

EXAMPLE 7

To test the effectiveness of siRNAs on inhibiting the ability of HCV to infect a healthy liver, potions of normal human liver are xenografted onto transgenic severe combined immunodeficient (SCID) mice according to methods well known to the skilled artisan.

Briefly, once the healthy liver has supplanted the mouse liver, liposome-encapsulated siRNA5, or control liposomes are administered by intravenous injection to the mice through the tail vein, or another accessible vein. The mice are dosed one time a day for 3-10 days. After the pre-dosing regimen, active HCV is then injected intravenously, or via hepatic injection, into the mice.

At about 6, 12, 18, 24 hours, and periodically up to about 5 days after the mice are infected with HCV, the mice are sacrificed and blood collected and the livers removed. The liver is divided into portions such that a portion is frozen using liquid nitrogen, a portion is fixed for paraffin embedding, and a portion is fixed for sectioning onto slides.

Using the appropriate allotment, HCV RNA is quantified using the TaqMan™ RNA assay kit previously utilized herein to determine the levels of HCV RNA in the liver cells. Further, anti-HCV antibody titers can be measured in the collected blood samples, along with serum ALT levels.

EXAMPLE 8

Modified siRNA can be prepared by chemical synthesis. In one embodiment, each C and U within a siRNA duplex, *e.g.* GL2, can be substituted with 2'-F-U and 2'F-C. To produce siRNA with 3'-end overhangs comprising 2'-F-U and 2'F-C, a universal support can be used. By selectively cleaving the oligo from the support, a practitioner can ensure that residues of the overhangs comprise modified nucleotides. Alternatively, the nucleotides comprising the 3'-end overhang can be unmodified dTdT.

2'-F RNA oligonucleotides can be synthesized on an Applied Biosystems 8909 or 8905 DNA/RNA synthesizer using the standard 1 μ mol beta-cyanoethyl phosphoramidite RNA chemistry protocol. The RNA phosphoramidite monomers and columns of Pac-A, 2'-F-Ac-C, iPr-Pac-G, 2'-F-U, and U-RNA CPG can be obtained from Glen Research (Sterling, VA). (See catalog nos. 10-3000-05, 10-3415-02, 10-3021-05, 10-3430-02, and 20-3430-41E, respectively.) Glen Research's Sulfurizing Reagent (catalog no. 40-4036-10) can be used as an oxidant to obtain a single phosphorothioate backbone between the 3' CPG and a subsequent base. To attain the coupling, the oxidizing step of the standard RNA 1 μ mol protocol can be replaced with the standard thioate 1 μ mol protocol. Cholesteryl-TEG phosphoramidite (Glen Research, catalog no. 10-1975-90) and cholestryl-TEG CPG (Glen Research, catalog no. 20-2975-41E) can be incorporated onto the 5' or 3' ends of one or more of the oligoribonucleotides. After synthesis, the 2'-F RNA's are cleaved and deprotected with 1:1 ammonium hydroxide/methylamine, and the silyl groups are removed with triethylamine trihydrofluoride using standard protocols. See e.g. http://www.glenres.com/productfiles/technical/tb_madeprotection.pdf. The oligoribonucleotides are then desalted on Sephadex G25 columns (Pharmacia NAP 25, catalog no. 17-08252-02) with sterilized water and purified using standard gel electrophoresis protocols. Modified siRNAs also can be obtained from commercial vendors such as Dharmacon (Lafayette, CO).

Alternatively, modified siRNA can be prepared by transcription using the Durascribe™ T7 Transcription Kit purchased from Epicentre Technologies (Madison, WI).

The modified siRNAs (dsRNAs) made by these methods contain phosphodiester linked oligonucleotides. Standard methods for making modified single-stranded RNAs, such as antisense molecules, are useful for making modified siRNAs, as modified single-stranded RNAs can be annealed together to form double stranded RNAs. Such standard methods include, but are not limited to, those described in Chiang *et al.*, *J.Biol.Chem.* 266, 18162-18171 (1991); Baker *et al.*, *J.Biol.Chem.* 272, 11994-12000 (1997); Kawasaki *et al.*, *J.Med.Chem.* 36, 831-841 (1993); Monia *et al.*, *J.Biol.Chem.* 268, 14514-14522 (1993).

EXAMPLE 9

To test whether siRNA directed to the HCV genome confers intracellular immunity against this human pathogen, a recently developed HCV cell culture systems in human hepatoma cell line, Huh-7, was used. One of the cell lines, 5-2, harbors autonomously replicating subgenomic HCV RNA (Bartenschlager, J. Virol, 2001). The subgenomic replicon carries firefly luciferase gene, allowing a reporter function assay as a measure of HCV RNA replication. Owing to cell culture adaptive mutations introduced into the genome, 5-2 cells replicate HCV RNA at levels of up to 5×10^4 virus particles/cell.

Using T7 transcription, several 21-bp siRNA duplexes against different regions of the 5'-UTR of the HCV genome were made. Briefly, two oligo double-stranded DNA molecules comprising the T7 promoter and the 5' UTR of HCV being oriented in either the sense direction or the antisense direction were generated. Each oligo DNA was then transcribed *in vitro* to produce (+) and (-) RNA and then treated with DNase I to remove the DNA template. The two RNA strands were allowed to anneal at 37°C overnight, generating dsRNA. After treating the dsRNA with RNase T1 to remove the unreacted ssRNA species, the dsRNA was purified for transfection.

Two exemplary modified siRNAs are provided below:

Chol-GL2 Chol-CGUACGCGGAAUACUUCGAUU
 UUGCAUGCGCCUUUAUGAAGCU

GL2 CGUACGCGGAAUACUUCGAUU
 UUGCAUGCGCCUUUAUGAAGCU

Each C and U within siRNA GL2, directed against the fruit fly luciferase gene, was substituted with 2'-F-U and 2'F-C. The modified siRNAs were transfected into the 5-2 cells using standard liposome transfection techniques. Specifically, the modified siRNAs were incubated for 4 hrs at 37°C in a 250 µl cell suspension containing 0.5 µl of Oligofectamine (Invitrogen, Carlsbad, CA), for 20 hrs in 375 µl serum containing culture medium, and for 24 hrs at 37°C in fresh medium without the

liposome-siRNA complex. Luciferase activity was measured 48 hours after transfection to determine the effect of the modified siRNAs on HCV replication.

Figure 11 shows that GL2 reduced the luciferase activity at increasing concentrations. Luciferase activity was reduced by 90% in cells transfected with 2'-F-GL2, but no significant reduction was seen in mocked transfected cells or with a control (2'-F-GFP=green fluorescent protein). The luciferase assay was carried out using a Luciferase assay system available from Promega Corp. (Madison, WI), according to the manufacturer's instructions.

The siRNA Chol-GL2 comprises a cholestry1 group on one of the 5' ends. 5-2 cells were incubated with various concentrations of Chol-GL2 in the absence of liposomes. Cells were harvested 48 hours after incubation and assayed for luciferase activity. Figure 12 shows that Chol-GL2 inhibited luciferase gene activity in a dose-dependent manner. InvA refers to chol-GL2 in inverted sequence.

EXAMPLE 10

To test the stability of 2' chemically modified siRNA compared to unmodified siRNA (siRNA), the following experiment is performed. Four nanograms of siRNA are added to a 20 μ L volume of 80% human serum from a healthy donor. This mixture is incubated at 37 C° for various times ranging from 1 minute up to 10 days. The results are depicted in lanes 2-10 of Figure 13. The same process is performed for 2' fluorine modified siRNA (2'-F siRNA) as well and the results are shown in lanes 12-20 and 22-25 of Figure 3. When the incubation process is finished, the mixtures are placed on ice and then immediately separated by PAGE along with a ³²P-siRNA control (See Lanes 1, 11 and 21 of Figure 13). The data show that the 2'-modified siRNA is stable over a period of 10 days as compared to unmodified siRNA.

EXAMPLE 11

To demonstrate the production of modified siRNA from long dsRNA, five micrograms of 1000 bp-long fluorinated dsRNAs (Figure 14, panel (A)) were incubated overnight with 15 units of human Dicer at 37°C. The resulting diced-

siRNAs were purified using a Sephadex G-25 column and electrophoresed on 20% PAGE (Figure 14, panel (B)). Figure 4 shows that recombinant human dicer effectively converts fluorinated-dsRNA into 2'F-siRNA.

EXAMPLE 12

To further test whether siRNAs directed to the HCV genome confer intracellular immunity against this human pathogen, the assay described in Example 1 was employed to test siRNAC1, siRNAC2, siRNA5B1, siRNA5B2, and siRNA5B4, each of which is shown in Figure 2. Each siRNA was tested at concentrations of 1 nM, 10nM and 100 nM. As shown in Figure 15, each of the siRNAs significantly inhibited luciferase activity in a dose-dependent manner. SiRNAC2 exhibited particular effectiveness.

EXAMPLE 13

As a follow-up to the experiments reported in Example 9, assays were performed to demonstrate that the cholesterol modification, and not the fluoro modification directed siRNA molecules to Huh-7 liver cells. Huh-7 cells were incubated with various concentrations of two kinds of Chol-GL2 siRNAs: one having a 2'-fluoro modification and the other lacking such a modification. The results, shown in Figure 16 demonstrate that the delivery of cholesterol-modified siRNA molecules to liver cells is due to the cholesterol, and not other modifications.

WHAT IS CLAIMED IS:

1. A method for inactivating a virus in a patient, comprising administering to said patient a modified siRNA in an effective amount to inactivate said virus.
2. The method of claim 1, wherein said modified siRNA is a 2' modified siRNA.
3. The method of claim 2, wherein the modification is at the 2' position of at least one ribonucleotide of said siRNA.
4. The method of claim 1, 2 or 3, wherein said modification is selected from the group consisting of fluoro-, methyl-, methoxyethyl- and propyl-modification.
5. The method of claim 4, wherein said fluoro-modification is a 2'-fluoro-modification or a 2',2'-fluoro-modification.
6. The method of any one of claims 1-5, wherein pyrimidines of said siRNA are modified, and said pyrimidines are cytosine, a derivative of cytosine, uracil, a derivative of uracil or a combination thereof.
7. The method of any one of claims 1-6, wherein both strands of said siRNA contain at least one modified nucleotide.
8. The method of any one of claims 1-7, wherein said virus is selected from the group consisting of hepatitis C virus (HCV), hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus, Ebola virus, influenza virus, rotavirus, reovirus, retrovirus, poliovirus, human papilloma virus (HPV), metapneumovirus and coronavirus.
9. The method of any one of claims 1-8, wherein said virus is hepatitis C virus.

10. The method of any one of claims 1-9, wherein said siRNA is prepared by
 - (a) identifying a target nucleotide sequence in an HCV genome for designing a small interfering RNA (siRNA); and
 - (b) producing an siRNA that has been modified to contain at least one modified nucleotide.
11. The method of any one of claims 1-9, wherein said siRNA is prepared by
 - (a) identifying a target nucleotide sequence in a virus genome for designing a small interfering RNA (siRNA); and
 - (b) producing an siRNA that has been modified to contain at least one modified nucleotide.
12. The method of claim 10, wherein said target nucleotide sequence is selected from the group consisting of 5'-untranslated region (5'-UTR), 3'-untranslated region (3'-UTR), core, and NS3 helicase.
13. The method of any one of claims 1-12, wherein said siRNA is siRNA5, siRNAC1, siRNAC2, siRNA5B1, siRNA5B2 or siRNA5B4.
14. An siRNA comprising a modified ribonucleotide, wherein said siRNA is resistant to RNase and retains the ability to inhibit viral replication.
15. The siRNA of claim 14, wherein said modified siRNA is a 2' modified siRNA.
16. The siRNA of claim 15, wherein the modification is at the 2' position of at least one ribonucleotide of said siRNA.

17. The siRNA of any one of claims 14-16, wherein the modification is selected from the group consisting of fluoro-, methyl-, methoxyethyl- and propyl-modification.
18. The siRNA of claim 17, wherein said fluoro-modification is a 2'-fluoro-medication or a 2',2'-fluoro-modification.
19. The method of any one of claims 14-18, wherein pyrimidines of said siRNA are modified, and said pyrimidines are cytosine, a derivative of cytosine, uracil, a derivative of uracil or a combination thereof.
20. The siRNA of any one of claims 14-19, wherein both strands of the siRNA contains modified nucleotides.
21. The siRNA of any one of claims 14-20, wherein said siRNA interacts with a target nucleotide sequence in a virus genome.
22. The siRNA of claim 21, wherein said virus is selected from the group consisting of hepatitis C virus (HCV), hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus, Ebola virus, influenza virus, rotavirus, reovirus, retrovirus, poliovirus, human papilloma virus (HPV), metapneumovirus and coronavirus.
23. The siRNA of claim 22, wherein said virus is hepatitis C virus (HCV).
24. A method of making a modified siRNA that targets a nucleic acid sequence in a virus comprising:
 - (a) preparing a modified-double stranded RNA (dsRNA) fragment containing at least one modified ribonucleotide in at least one strand that spans the genome of a target agent; and

(b) cleaving said modified-dsRNA fragments with recombinant human Dicer resulting in more than one modified siRNA.

25. The method of claim 24, further comprising:

(c) isolating said modified siRNAs.

26. The method of claim 24 or 25, wherein said target agent is a virus.

27. The method of claim 26, wherein said virus is selected from the group consisting of hepatitis C virus (HCV), hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus, Ebola virus, influenza virus, rotavirus, reovirus, retrovirus, poliovirus, human papilloma virus (HPV), metapneumovirus and coronavirus.

28. A method for inactivating a virus in a patient comprising administering to said patient a modified siRNA consisting of about 10 to about 30 ribonucleotides in an effective amount to inactivate said virus.

29. The method of claim 28, wherein said modified siRNA consists of about 19 to about 23 ribonucleotides.

30. The method of claim 28 or 29, wherein said modified siRNA is a 2' modified siRNA.

31. The method of any one of claims 28-30, wherein the modification is at the 2' position of at least one ribonucleotide of said siRNA.

32. The method of any one of claims 28-31, wherein said modification is selected from the group consisting of fluoro-, methyl-, methoxyethyl- and propyl-modification.

33. The method of claim 32, wherein said fluoro-modification is a 2'-fluoro-modification or a 2',2'-fluoro-modification.

34. The method of any one of claims 28-33, wherein pyrimidines of said siRNA are modified and said pyrimidines are cytosine, a derivative of cytosine, uracil, a derivative of uracil or a combination thereof.

35. The method of any one of claims 28-34, wherein both strands of said siRNA contain modified nucleotides.

36. The method of any one of claims 28-35, wherein said virus is selected from the group consisting of hepatitis C virus (HCV), hepatitis A virus, hepatitis B virus, hepatitis D virus, hepatitis E virus, Ebola virus, influenza virus, rotavirus, reovirus, retrovirus, poliovirus, human papilloma virus (HPV), metapneumovirus and coronavirus.

37. The method of any one of claims 28-36, wherein said virus is hepatitis C virus (HCV).

38. The method of any one of claims 28-37, wherein said siRNA is prepared by

(a) identifying a target nucleotide sequence in a HCV genome for designing a small interfering RNA (siRNA); and

(b) producing an siRNA that has been modified to contain at least one modified nucleotide.

39. The method of any one of claims 28-37, wherein said siRNA is prepared by

(a) identifying a target nucleotide sequence in a virus genome for designing a small interfering RNA (siRNA); and

(b) producing an siRNA that has been modified to contain at least one modified nucleotide.

40. The method of claim 38, wherein said target nucleotide sequence comprises a conserved nucleotide sequence necessary for HCV replication.

41. The method of claim 40, wherein said conserved nucleotide sequence is selected from the group consisting of 5'-untranslated region (5'-UTR), 3'-untranslated region (3'-UTR), core, and NS3 helicase.

42. The method of any one of claims 28-41, wherein said siRNA is siRNA5, siRNAC1, siRNAC2, siRNA5B1, siRNA5B2 or siRNA5B4.

43. A double-stranded RNA molecule of from about 10 to about 30 nucleotides that inhibits replication of hepatitis C virus (HCV).

44. The double-stranded RNA molecule of claim 43 comprising a nucleotide sequence at least 80% identical to the nucleotide sequence of siRNA5, siRNAC1, siRNAC2, siRNA5B1, siRNA5B2 or siRNA5B4.

45. A method of inducing targeted RNA interference toward HCV in hepatic cells, comprising administering the double-stranded RNA molecule of claim 43 or 44 to hepatic cells, wherein the nucleotide sequence of said double-stranded RNA molecule corresponds to an HCV nucleotide sequence.

46. A method of inhibiting replication of hepatitis C virus (HCV), comprising administering the RNA polynucleotide molecule of claim 43 or 44 to cells infected with HCV.

47. A vector comprising a DNA segment encoding the RNA molecule of claim 43 or 44.

48. The vector of claim 47, wherein the sense strand of said double-stranded RNA molecule is operably linked to a first promoter and wherein the antisense strand of said double-stranded RNA molecule is operably linked to a second promoter.

49. The vector of claim 48, wherein said first and second promoters are selected from the group consisting of U6 and H1.

50. The vector of claim 48 or 49 wherein said first and second promoters are the same.

51. The vector of claim 47, wherein the sense and antisense strands of said RNA molecule are under the control of a single promoter.

52. The vector of claim 51, wherein said single promoter is selected from the group consisting of U6 and H1.

53. A host cell comprising the vector of any one of claims 47-52.

54. A method of inhibiting replication of hepatitis C virus (HCV) in cells carrying HCV, comprising transfecting said cells with the vector of any one of claims 47-52.

55. A method of treating hepatitis C in a subject in need thereof, comprising administering a composition comprising the RNA molecule of claim 43 or 44 to said subject.

56. A method of treating hepatitis C in a subject in need thereof, comprising administering the vector of any one of claims 47-52 to said subject.

57. A modified siRNA molecule, comprising a double-stranded RNA molecule of from about 10 to about 30 nucleotides in length, which mediates RNA interference toward a target agent or virus, and which is linked to at least one receptor-binding ligand.

58. The modified siRNA molecule of claim 57, wherein said receptor-binding ligand is attached to a 5' -end or 3' -end of said siRNA molecule.

59. The modified siRNA molecule of claim 57 or 58, wherein said receptor binding ligand is attached to multiple ends of said siRNA molecule.

60. The modified siRNA molecule of any one of claims 57-59, wherein said receptor-binding ligand is selected from the group consisting of a cholesterol, an HBV surface antigen, low-density lipoprotein, an HIV-1 surface antigen, an influenza virus surface antigen, an RSV surface antigen, an HPV surface antigen and a polio virus surface antigen.

61. The modified siRNA molecule of any one of claims 57-60, wherein said receptor-binding ligand is cholesterol.

62. The modified siRNA molecule of any one of claims 57-61, further comprising a modification at the 2' position of at least one ribonucleotide, which modification at the 2' position of at least one ribonucleotide renders said siRNA resistant to degradation.

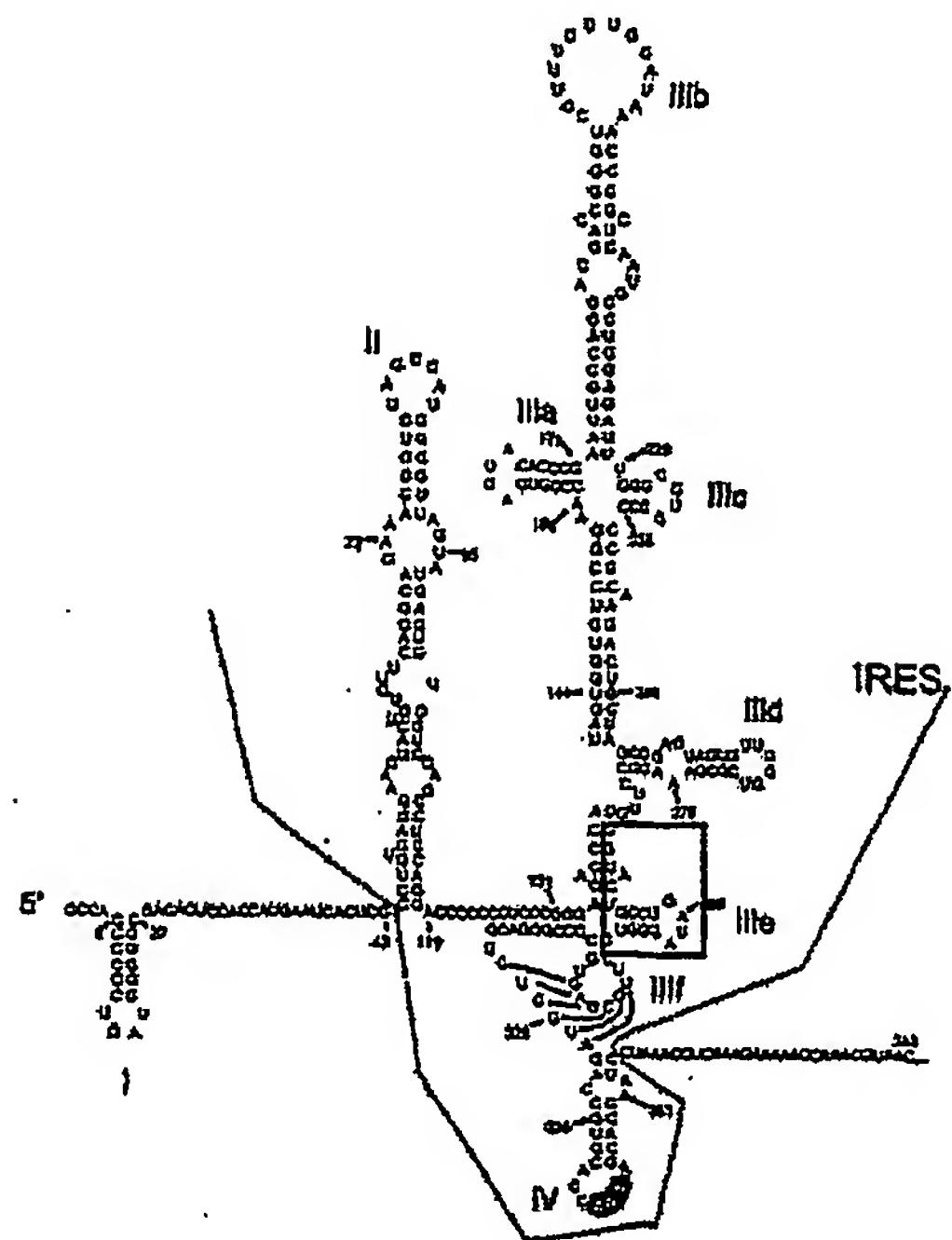
63. The modified siRNA molecule of claim 62, wherein said modification at the 2' position of at least one ribonucleotide is a 2'-fluoro-modification or a 2',2'-fluoro-modification.

64. A method of inducing targeted RNA interference in a patient, comprising administering to said patient an effective amount of the siRNA of any one of claims 57-63.

65. A method of inducing targeted RNA interference in a patient, comprising administering to said patient an effective amount of the siRNA of claim 61.

66. A method of inducing targeted RNA interference in a patient, comprising administering to said patient an effective amount of the siRNA of claim 63.

Fig. 1. The Sequence and Secondary Structure of 5'UTR of HCV Genome.



The region where siRNAs was designed is boxed.
The sequence of the 21-bp siRNA₅ is shown below.

siRNA ₅	286	304
	5'-GUACUGCCUGAUAGGGUGCUU	
	UUCAUGACGGACUAUCCCACG-5'	
GL2	5'-CGUACGCGGAUACUUCGAUU	
	UUGCAUGCGCCUUUAUGAAGCU-5'	
GL3	5'-CUUACGCGUGAGUACUUCGAUU	
	UUGAAUGCGACUCAUGAAGCU--5'	
SIN	5'-AUCUCUACGGUGGUCCUAUU	
	UUUAGAGAUGCACCAGGAUU--5'	

2/24
Fig. 2

	Domain	sequence (NN-N19-NN)	Position			
5U8	5'UTR	cc-CUGUGAGGAACUACUGUCU-uc	45-63	sense	CUGUGAGGAACUACUGUCUUC	
				antisense	AGACAGUAGUUCUCACAGGG	
5U9	5'UTR	ua-CUGUCUUCACGCAGAAAGC-gu	58-76	sense	CUGUCUUCACGCAGAAAGCGU	
				antisense	GCUUUCUGCGUGAAGACAGUA	
5U10	Core	cg-AGACUGCUAGCCGAGUAGU-gu	244-262	sense	AGACUGCUAGCCGAGUAGUUGU	
				antisense	ACUACUCGGCUAGCAGUCUCG	
C1	Core	ga-AUCCUAAACCUCAAAGAAA-aa	352-370	sense	AUCCUAAACCUCAAAGAAAAAA	
				antisense	UUUCUUUGAGGUUUAGGAUUC	
C2		gg-UCAGAUCGUCGGUGGAGUU-ua	425-443	sense	UCAGAUCGUCGGUGGAGUUUA	
				antisense	AACUCCACCGACGAUCUGACC	
C3		gg-UAAGGUCAUCGAUACCCUC-ac	701-719	sense	UAAGGUCAUCGAUACCCUCAC	
				antisense	GAGGGUAUCGAUGACCUCUACC	
C4		ac-GGCGUGAACUAUGCAACAG-gg	822-840	sense	GGCGUGAACUAUGCAACAGGG	
				antisense	CUGUUGCAUAGUUCACGCCGU	
C5		cc-GGUUGCUCCUUUCUAUCU-uc	852-870	sense	GGUUGCUCCUUUCUAUCUUC	
				antisense	AGAUAGAAAAGGAGCAACCGG	
5B1	NS5B	gc-UCUCAUACGGAUUCCAAU-ac	8163-8181	sense	UCUCAUACGGAUUCCAAUAC	
				antisense	AUUGGAAUCCGUAGAAGAGC	
5B2		ca-UACGGAUUCCAAUACUCUC-cu	8167-8187	sense	UACGGAUUCCAAUACUCUCU	
				antisense	GAGAGUAUUGGAAUCCGUAG	
5B3		uu-UGACUCAACGGUCACUGAG-aa	8270-8288	sense	UGACUCAACGGUCACUGAGAA	
				antisense	CUCAGUGACCGUUGAGUCAA	
5B4		cc-UUCACGGAGGCUAUGACUA-ga	8613-8631	sense	UUCACGGAGGCUAUGACUAGA	
				antisense	UAGUCAUAGCCUCCGUGAAGG	
5B5		au-ACGACUUGGAGJUGAUAAAC-au	8671-8689	sense	ACGACUUGGAGUUGAUAAACAU	
				antisense	GUUAUCAACUCCAAGUCGUAU	
5B6		au-UCCUGGUAGGCAACAUCA-uc	8817-8835	sense	UCCUGGUAGGCAACAUCAUC	
				antisense	UGAUGUUGCCUAGCCAGGAAU	
5B7		uu-GUGGCAAGUACCUCUCAA-cu	9160-9178	sense	GUGGCAAGUACCUCUCAACU	
				antisense	UUGAAGAGGUACUUGCCACAA	
5B8		au-GUGGUGCCUACUCCUACUU-uc	9317-9335	sense	GUGGUGCCUACUCCUACUUUC	
				antisense	AAGUAGGAGUAGGCACCACAU	
3U1	3'UTR	cu-UUGGUGGUCCAUCAUUAGC-cc	9506-9524	sense	UUGGUGGUCCAUCAUUAGCCC	
				antisense	GCUAAGAUGGAGCCACCAAAG	
3U2		gu-CACGGCUAGCUGUGAAAGG-uc	9531-9549	sense	CACGGCUAGCUGUGAAAGGUC	
				antisense	CCUUCACAGCUAGCCGUGAC	
3U3		ag-CCGCUUGACUGCAGAGAGU-gc	9558-9576	sense	CCGCUUGACUGCAGAGAGUGC	
				antisense	ACUCUCUGCAGUCAAGCGGCU	

Fig. 3

1 ttatttagtt ttacacctacc cagggaaaagc caaccaaccc cgatcttgc tagatctgtt
61 ctctaaacga actttaaaat ctgtgttagct gtcgctcggt tgcatgccta gtgcaccc tac
121 gcagtataaaa caataataaaa tttactgtc gtgtacaaga aacgagtaac tcgtcccct
181 tctgcagact gcttacgggt tcgtccgtgt tgcatgtcat catcagcata cctaggttc
241 gtccgggtgt gaccgaaagg taagatggag agcctgttc tgggtgtcaa cgagaaaaaca
301 cacgtccaaac tcaagttgcc tgccttcag gttagagacg tgctagtgcg tggctcggg
361 gactctgtgg aagaggccct atcggaggca cgtgaacacc tcaaaaaatgg cacttgtgtt
421 ctagtagagc tggaaaaaagg cgtactgccc cagctgaac agccctatgt gtccattaaa
481 cgttctgtatg ccttaagcac caatcaccgc cacaaggctcg ttgagctggt tgcaaaaaatg
541 gacggcattc agtacggctcg tagcggtata acactgggg tactcgtgcc acatgtggc
601 gaaaccccaa ttgcataccg caatgttctt ctgcgtaaaga acggtataaa gggagccggt
661 ggtcatacgat atggcatcga tctaaagtct tatgacttag gtgacgagct tggactgtat
721 cccatgtaaag attatgtaaaca aaactggaaac actaaggcatg gcagtgggtc actccgtgaa
781 ctcactcgtg agctcaatgg aggtgcagtc actcgctatg tgcacaacaa ttctgtggc
841 ccagatgggt acccttcttga ttgcataaaa gatttctcg cacgcgcggg caagtcaatg
901 tgcactctt ccgaacaaact tgattacatc gagtcgaaga gaggtgtcta ctgcgtccgt
961 gaccatgagc atgaaattgc ctggttcact gagcgctctg ataagagcta cgagcaccag
1021 acacccttcg aaattaagag tgccaagaaaa ttgacactt tcaaaggggaa atgccccaaag
1081 ttgtgtttc ctcttaactc aaaagtcaaa gtcattcaac cacgtgttga aaagaaaaag
1141 actgaggggt tcatggggcg tatacgctct gtgtaccctt tgcatctcc acaggagtgt
1201 aacaatatgc acttgtctac ctgtatgaaa tgaatcatt gcatgtaaatgttcatggcag
1261 acgtgcact ttctgtaaaggc cacttgtgaa cattgtgca ctgtaaaatgtt agttatgtaa
1321 ggacctacta catgtgggtt cctacactt aatgtgttag tggaaatgcc atgtccfgcc
1381 tgtcaagacc cagagattgg acctgagcat agtgtgtcag attatcacaac ccactcaaac
1441 attgaaactc gactccgcaaa gggaggttagg actagatgtt tggaggctg tgggttgcc
1501 tatgtggct gctataataa gcgtgcctac tgggttcctc gtgtactgtc tggatattggc
1561 tcaggccata ctggcaitac tggtgacaat gtggagacat tgaatgagga tctccctgag
1621 atactgagtc gtjaacgtgt taacattaaac attgtggcg atttcattt gaatgaagag
1681 gttggccatca ttggccatc ttctctgtct tctacaatgtt ccttattgtt cactataaaag
1741 agtcttgatt acaagtctt caaaaccatt gtgtgtccct gcggttaacta taaaggtaacc
1801 aaggggaaagc ccgtaaaagg tgcttggaaac attggacaac agagatcaat ttaacacca
1861 ctgtgtgggtt ttccctcaca ggctgtgggtt gtatcagat caattttgc ggcacactt
1921 gatgcagcaa accactcaat tccgtatttgc caaagagcag cgtcaccat actgtatgtt
1981 atttctgtac acgttacatc tcttgtcgtac gccatggttt atttctgtac cctgtccacc
2041 aacagtgta ttattatggc atatgttaact ggtggtctt tacaacagac ttctcgtgg
2101 ttgtcttaatc ttgggcac tactgtgaa aaactcagggc ctatcttgc atggatttag
2161 gcgaaaactttaatc gtgcaggagt tgaatttctc aaggatgtt gggagattct caaatatttctc
2221 attacaggtt ttttgacat cgtcaagggtt caaatacagg tgcgttcaga taacatcaag
2281 gatgtgttaa aatgttcat tgaatgtttt aacaaggcac tgcgttgcattt cattgtatcaa
2341 gtcactatcg ctggcgcaaa gtgtgttgcata ctcaacttagt gtgtgttgc ttcgtccaa
2401 agcaaggac ttaccgtca gtgtatgtt ggcaaggagc agctgtcaactt actcatgtt
2461 cttaaaggcac caaaagaatgtt aacctttctt gaagggttgcattt cattgtatcaa
2521 tctgtggggg ttgttcttcaaa gaacgggttgcata ctcaacttagt gtgtgttgc ttcgtccaa
2581 ttcacaaatgtt gagctatgtt cggcacacca gtgtgttgcata atggcctcat gtcgttgc
2641 attaaggaca aagaacaata ctgcgttgcattt tctccttgcattt tactgtgttgcattt
2701 ttgcgttgc accaattttttt ggtgttgcattt ttggagaaga tactgtgttgcattt
2761 gaaggtaatc gttacaagaa tttgtgttgcattt acattgtgttgcattt ttgtgttgcattt

2821 gtgcttaatg aaaagtgctc tgtctacact gttgaatccg gtaccgaagt tactgagtt
2881 gcatgttgt tagcagaggc tgggtgaag actttacaac cagttctga ttccttacc
2941 aacatggta ttatctga tgagtggagt gtagctacat tctacttatt tgatgatgct
3001 ggtgaagaaa actttcatc acgtatgtat tttcccttt accctccaga tgaggaagaa
3061 gaggacgatg cagagtgtga ggaagaagaa attgtatgaaa cctgtgaaca tgagtacgg
3121 acaggaggatg attatcaagg ttcctctg gaatttgggt cctcagctga aacagttcg
3181 gttgaggaag aagaagagga agactggctg gatgatacta ctgagcaatc agagattgag
3241 ccagaaccag aacctacacc tgaagaacca gttaatcagt ttactgtt ttaaaactt
3301 actgacaatg ttgccattaa atgtgtgac atcgtaagg aggcacaaag tgctaatcct
3361 atgggattt taaatgctgc taacatacac ctgaaacatg gtgggtgtt agcagggtgca
3421 ctcaacaagg caaccaatgg tgccatgcaa aaggagagtg atgattacat taagctaaat
3481 ggcctctt cagtaggagg gtctgttg cttctggac ataatctgc taagaagtgt
3541 ctgcattgtt tggacctaa cttaaatgca ggtgaggaca tccagctt taaggcagca
3601 tataaaaatt tcaattcaca ggacatctt cttgcaccat tttgtcagc aggcatattt
3661 ggtctaaac cacttcagtc ttacaaatgt tgctgcaga cggttcgtac acaggttat
3721 attgcagtca atgacaaagc tcttatgag caggttgtca tggattatct tgataacctg
3781 aagcctagag tggaaagcacc taaacaagag gagccaccaa acacagaaga ttccaaaact
3841 gaggagaaat ctgtcgtaca gaagcctgtc gatgtgaagc caaaaattaa ggcctgcatt
3901 gatgaggtt ccacaacact ggaagaaact aagttctt ccaataagtt actcttgg
3961 gctgatatca atggtaagct ttaccatgt ttcagaaca tgcttagagg tgaagatatg
4021 ttttccttg agaaggatgc accttacatg gtaggtgtatg ttatcactag tggatgtatc
4081 acttgtgtt taataccctc caaaaaggct ggtggacta ctgagatgtc ctcaagagct
4141 ttgaagaaag tgccagtttga tgagtatata accacgtacc ctggacaagg atgtgttgt
4201 tatacacttg aggaagctaa gactgtctt aagaaatgca aatctgcatt ttatgtacta
4261 ctttcagaag cacctaattgc taaggaagag attcttagaa ctgtatctg gaatttgaga
4321 gaaatgttg ctcatgctga agagacaaga aaattaatgc ctatatgcatt ggatgttaga
4381 gccataatgg caaccatcca acgtaagtat aaaggaatta aaattcaaga gggcatcg
4441 gactatggtg tccgattctt ctttatact agttaagagc ctgtacttc tattattacg
4501 aagctgaact ctctaaatga gccgttgc acaatgccaa tggttatgt gacacatgt
4561 tttaatcttg aagaggctgc gcgtgtatg ctttccttta aagctcctgc cgtgtgtca
4621 gtatcatcac cagatgctgt tactacataat aatggataacc tcacttcgtc atcaaagaca
4681 tctgaggagc acttgttaga aacagttct tggctggct ttacagaga tggccttat
4741 tcaggacagc gtacagagtt aggtgttga ttcttaagc gtggtgacaa aattgtgtac
4801 cacactctgg agagccccgt cgagttcat ttgacggtg aggtcttcc acttgacaaa
4861 ctaaagagtc tttatccct gggggagggt aagactataa aagtgttac aactgtggac
4921 aacactaattccacaca gttgtggat atgtctatga cataggaca gcagtttgt
4981 ccaacatact tggatggtgc tgatgtaca aaaatcaaact cttatgtaaa tcatgagggt
5041 aagactttct ttgtactacc tagtgtatgac acactacgtt gtgaagctt cgagtactac
5101 catactcttg atgagagtt ttttgtttagg tacatgtctg tttaaaccacaca
5161 tggaaatttc ctcaagttgg tggtttaact tcaattaaat gggctgataa caattgttat
5221 ttgtctatgt ttttattagc acttcaacag ctgttgtca aattcaatgc accagcactt
5281 caagaggctt attatagagc ccgtgttgtt gatgtgtca acttttgtgc actcataact
5341 gtttacagta ataaaaactgt tggcgagctt ggtgttgtca gagaaactat gacccatctt
5401 ctacagcatg ctaatttggaa atctgcaaaag cgagttcttta atgtgggtgtt taaacattgt
5461 ggtcagaaaa ctactacctt aacgggtgttga gaagctgtga tgtatatggg tactctatct
5521 tatgataatc ttaagacagg tttccattt ccatgtgtgtt gtggctgtca tgctacacaa
5581 tatctatgtac aacaagagtc ttctttgtt atgtgtctg caccacctgc tgagtataaa
5641 ttacagcaag gtacattctt atgtgttgtt ggtgttgtt gtttttgttca gtttttgtt
5701 tacactcata taactgttca ggagaccctc tatgttattt acggagctca ctttacaaag
5761 atgtcagagt acaaaggacc agtgtactgtt gtttttgttca aggaaacatc ttacactaca
5821 accatcaagc ctgtgttgtt taaactgttca gggttgtt acacagagat ttttttgtt
5881 tggatgggtt attataaaa ggataatgtt tactatacag agcaggctt agacccttgt
5941 ccaactcaac ctttacaaa tggcgatgtt gataatttca aactcacatg ttcttacacaa
6001 aaatttgttca atgatttaaa tcaaatgttca ggcttcacaa agccagctt acggagagctt
6061 ttttttgttca ttttttgttca ttttttgttca ttttttgttca ttttttgttca
6121 tcaqccqagtt tcaagaaaagg ttttttgttca ttttttgttca ttttttgttca ttttttgttca

6181 caggctacaa ccaagacaac gttcaaaccā aacacttgtt gttacgttg tcttggagt
6241 acaaagccag tagatacttc aaattcattt gaagttctgg cagtagaaga cacacaagga
6301 atggacaatc ttgcttgc aagtaacaaa cccacctcg aagaagtgtt ggaaaatcc
6361 accatacaga aggaagtcat agagtgtcac gtgaaaacta ccgaagtgtt aggcaatgtc
6421 atacttaaac catcagatga aggtgttaaa gtaacacaag agtaggtca tgaggatct
6481 atggctgctt atgtggaaaa cacaagcatt accattaaga aacctaata gcttcacta
6541 gccttaggtt taaaaacaat tgccactcat gtttgcgtt caattaatag tttccitgg
6601 agtaaaaattt tggcttatgt caaaccattc ttaggacaag cagcaattac aacatcaaatt
6661 tgcgctaaga gattagcaca acgtgtgtt aacaattata tgccttatgtt gttacatta
6721 ttgtccaat tttgtactt tactaaaagt accaattcta gaatttagac ttcaattac
6781 acaactattt cttaaaaatag tttttttttt gttttttttt tttttttttt tttttttttt
6841 aattatgtga agtcacccaa attttctaaa ttgttccaa tcgctatgtt gctattttttt
6901 ttaagtattt gcttaggttc tctaattttt gtaactgtt gttttttttt actttttatct
6961 aattttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7021 gttactacta tggattttt tttttttttt tttttttttt tttttttttt tttttttttt
7081 gactcccttg attttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7141 ctagacttga caattttttt tttttttttt tttttttttt tttttttttt tttttttttt
7201 aaattttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7261 agtcatttca ttagcaattt tttttttttt tttttttttt tttttttttt tttttttttt
7321 cccgtttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7381 agctatgttc atatcatgga tggtttgcacc tttttttttt tttttttttt tttttttttt
7441 aatcggttcca cacgcgttga gtgtacaact attttttttt tttttttttt tttttttttt
7501 gtctatgcaaa atggaggccg tggcttctgc aagactcaca attttttttt tttttttttt
7561 gacacattttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7621 cagttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7681 gtgaaaaatgtt ggcgcgttca cttttttttt tttttttttt tttttttttt tttttttttt
7741 catccgttca cccattttt tttttttttt tttttttttt tttttttttt tttttttttt
7801 ctgcctttaa atgtcatatg tttttttttt tttttttttt tttttttttt tttttttttt
7861 tctgtttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7921 ctgttatcatg acgtttttttt tttttttttt tttttttttt tttttttttt tttttttttt
7981 gacacattttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8041 gctcacagcg agttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8101 gctgccccac aaggtttttt tttttttttt tttttttttt tttttttttt tttttttttt
8161 aaactttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8221 acctataata aggtttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8281 gcaaggcata tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8341 aaagactaca tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8401 aacaacatac tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8461 actaaaatctt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8521 gcccattttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8581 ttgtcaatcc atgtttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8641 gtcactcgatg acatcattt tactgtatgtt tttttttttt tttttttttt tttttttttt
8701 gcatggtttca gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8761 gctatcattt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8821 gcaatcaatg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8881 tgctacacac tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
8941 gctgtttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9001 actaattttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9061 cttatggatg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9121 gtaacaactt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9181 attttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9241 ggagttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9301 caacccgttgg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9361 ttgttgcattt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9421 catgtttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
9481 ccagtttaca gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt

12901 aacctaaata gaggtatggc gctgggcagt ttagctgcta cagtagtct tcaggctgga
12961 aatgctacag aagtacctgc caattcaact gtgccttcct tctgtgctt tgcatctac
13021 cctgctaaag catataagga ttacctagca agtggaggac aaccaatcac caactgtgt
13081 aagatgtgt gtacacacac tggcacagga caggcaatta ctgtaacacc agaagctaac
13141 atggaccaag agtccttgg tggcttca tttgtctgt atttagatg ccacattgac
13201 catccaaatc ctaaaggatt ctgtacttg aaaggtaagt acgtccaaat acctaccact
13261 tgtgctaatt acccagtggg tttacactt agaaaacacag tctgtaccgt ctgcggaaatg
13321 tggaaagggtt atggctgttag ttgtgaccaa ctccgcgaac cctgtatgca gtctgcggat
13381 gcatcaacgt tttaaacgg gttgcggtg taagtgcagc ccgtcttaca ccgtgcggca
13441 caggcactag tactgtatgtc gtctacaggg ctttgatatttacaacgaa aaagttgctg
13501 gtttgcaaa gttcctaaaa actaattgtt gtgcctcca ggagaaggat gaggaaggca
13561 atttattaga ctcttacttt gtagttaaga ggcatactat gtctaaactac caacatgaag
13621 agactattta taacttgggtt aaagattgtc cagcggtgc tgtccatgac ttttcaagt
13681 ttagagtaga tggctgacatg gtaccacata tatcagtc ggcgtactt aaatacacaa
13741 tggctgattt agtctatgtc ctacgtcatt ttgtgaggg taattgtat acattaaaag
13801 aaatacttgt cacatacaat tgctgtatg atgattattt caataagaag gattggatg
13861 acttcgtaga gaatccgtac atcttacgcg tatatgctaa cttaggtgag cgtgtacgcc
13921 aatcattattt aaagactgtt caattctgcg atgctatgcg tgatgcaggc attgtaggcg
13981 tactgacatt agataatcag gatcttaatg ggaactggta cgattcggg gatttcgtac
14041 aagtagcacc aggctgcggaa gttccttattt gggattcata ttactcatgt ctgtatgccc
14101 tcctcacttt gactaggc ttggctgctg agtcccattt ggtgtctgtat ctgcacaaac
14161 cacttattaa gtgggattt gtaaatatg atttacggaa agagagactt tgctcttcg
14221 accgttattt taaatattgg gaccagacat accatcccaa ttgtttaac tttttggatg
14281 ataggtgtat ctttactgtt gcaaaacttta atgtgttatt ttctactgtg ttccaccta
14341 caagtttgg accacttagta agaaaaatattt tagtagatgg ttgttccctttt gtttttca
14401 ctggatacca ttttcgttag ttaggagtcg tacataatca ggtgtaaac ttacatagct
14461 cgcgtctcag ttcaaggaa cttttagtgtt atgctgtga tccagctatg catcagcatt
14521 ctggcaattt attgcttagat aaacgcacta catgttttc agtagctgca ctaacaaaca
14581 atgttgctt tcaaactgtc aaacccggta atttaataa agactttat gacttgctg
14641 tgcctaaagg ttcttaag gaaggaagt ctgtgaact aaaacacttc ttcttgctc
14701 agatggcaaa cgctgtatc agtgtttatg actattatcg ttataatctg ccaacaatgt
14761 gtgatatcag acaactccta ttctgtttt aagttgtga taaatactttt gattgttacg
14821 atggtggctg tattaatgcc aaccaagtaa tcgttaacaa tctggataaa tcagctgggt
14881 tcccatattaa taaatgggtt aaggcttagac ttattatga ctcaatgat tatgaggatc
14941 aagatgcact ttgcgttat actaagcgta atgtcatccc tactataact caaatgaatc
15001 ttaagtatgc cattatgtca aagaatagag ctgcaccgt agctgggttc tctatctgt
15061 gtactatgac aaatagacag ttcatcaga aatttttggaa gtcaatagcc gccactagag
15121 gagctactgt ggtatttggaa acaagcaagt ttacgggtgg ctggcataat atgtttaaaa
15181 ctgttacag tgatgttagaa actccacacc ttatgggttg ggattatcca aaatgtgaca
15241 gagccatgcc taacatgtt aggataatgg cctctttgt tctgtctgc aaacataaca
15301 ctgtgtttaa ctatcacac cgtttctaca ggttagctaa cgagtgtgcg caagtattaa
15361 gtgagatgtt catgtgtggc ggctcactat atgttttttcc accgtggaaaca tcatccgggt
15421 atgctacaac tgcttactgtt aatagtgtct ttaacatttg tcaagctgtt acagccatgt
15481 taaatgcact tcttcaact gatggtaata agatagctga caagtatgtc cgcaatctac
15541 aacacaggct ctatgttgtt ctctatagaa ataggatgt tgatcatgaa ttctgtggatg
15601 agttttacgc ttacctgcgt aaacatttct ccatgtatgt tctttctgtt gatgccgttg
15661 tgcgtataa cagtaactat gcggctcaag gtttagtagc tagcatataa aacttttaagg
15721 cagttctta ttatcaaaat aatgtgttca tgcgtggc aaaaatgtgg actgagactg
15781 accttactaa aggacctcac gaattttgtt cacagcatac aatgttagtt aaacaaggag
15841 atgattacgt gtacctgcct taccctggatc catcaagaat attaggcgca ggctgttttgg
15901 tcgtatgtat tgcctaaaca gatgggtacac ttatgttga aaggttcgtt tcaactggct
15961 ttgtatgttta cccacttaca aaacatccta atcaggatgt tgcgtatgtc ttctactgt
16021 atttacaata cattagaaag ttacatgtatg agcttactgg ccacatgtt gacatgttatt
16081 ccgtaatgtt aactaatgtt aacacccac ggtactggaa acctgagttt tatgaggatc
16141 tgcgtatgtt acatacgtt tgcaggctt taggtgtttt tgcgtatgtc aattcacaga
16201 ctgcacttcg ttgcgggtgcc tgcgtatgtt gaccattccct atgttgcgtt tgctgtatgt

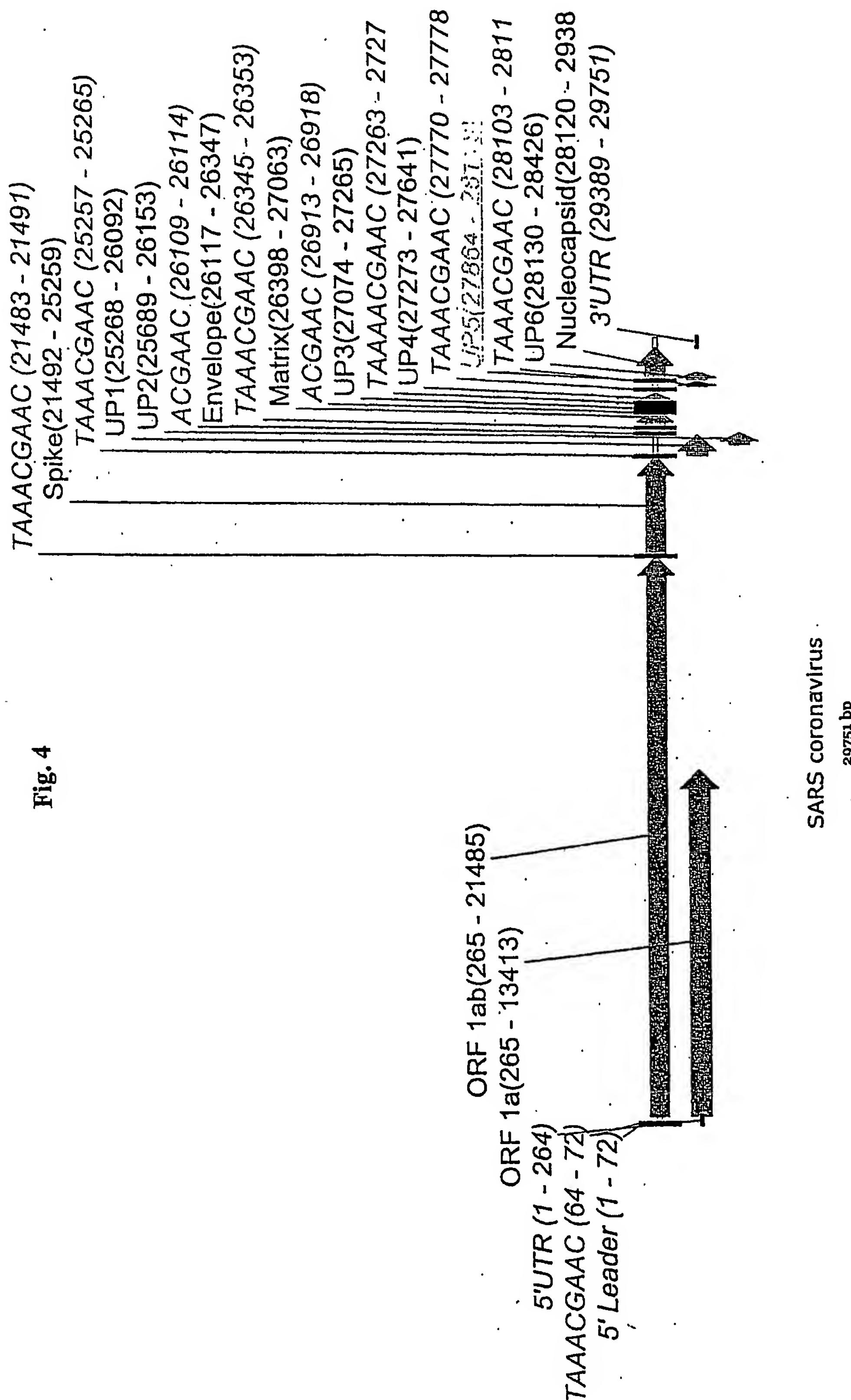
16261 accatgtcat ttcaacatca cacaattag ttttgttgc taatccctat gtttgcata
16321 ccccagggtt tgatgtcact gatgtgacac aactgtatct aggaggtatg agctattatt
16381 gcaagtca ca taaggctccc attagtttc cattatgtgc taatggtcag gttttgggt
16441 tataaaaaaa caccatgtgt a ggcagtgaca atgtcactga cttaatgcg atagcaacat
16501 gtgattggac taatgtggc gattacatac ttgccaacac ttgtactgag agactcaagc
16561 tttcgacagc agaaacgctc aaagccactg aggaaacatt taagctgtca tatggatttg
16621 ccactgtacg cgaagtactc tctgacagag aattgcatac ttcatggag gttggaaaac
16681 ctagaccacc attgaacaga aactatgtct ttactggta ccgtgttaact aaaaatagta
16741 aagtacagat tggagagatc acctttgaaa aagggtgacta ttgtgtatc ttgtgtaca
16801 gaggtactac gacatacaag ttgaatgtt gtgattactt ttgtgtaca tctcacactg
16861 taatgccact tagtgcaccc actctatgtc cacaagagca ctatgtgaga attactggct
16921 tgtacccaa acactcaacatc tcagatgatg tttctagcaa ttgtgtcaat tatcaaaagg
16981 tcggcatgca aaagtactct acactccaag gaccacccgg tactggtaag agtcatttt
17041 ccatcgact tgctcttat tacccatctg ctgcatactg ttatccatg tgctctatg
17101 caagctgtga tgccctatgt gaaaaggcat taaaatattt gcccatagat aatgtatgta
17161 gaatcatacc tgcgcgtgcg cgcgttagat ttgtgtatc ttgtgtaca aatcaacac
17221 tagaacatgtt ttgtgtatc actgtttatc cattgttgcg aacaactgtt gacattgttag
17281 ttgtgtatc aatctatg gctactaattt atgtgtatc ttgtgtatc gctagacttc
17341 gtgcacccaa ctcacgtctat attggcgtat ctgcataattt accagcccccc cgacatgtc
17401 tgactaaagg cacactagaa ccagaatattt ttaatttcgtt gtgcagactt atgaaaacaa
17461 taggtccaga catgtccctt ggaactgtc gcccgtgtcc tgctgtatc ttgtgtatc
17521 tgatgtatc aataagctaa aagcacacaa ggataagtca gtcataatgt
17581 tcaaaatgtt ctacaaaggat ttattacac atgtgtttc atctgcatac aacagaccc
17641 aataaggctt tgtaagagaa ttcttacac gcaatccgtc ttggagaaaa gctgttttta
17701 ttcacccatc taatttccatc aacgtgtatc ttcaaaaaat cttagatttgc cttacgcaga
17761 ctgtgtatc atcacagggt tctgtatc actatgtatc attcacacaa actactgaaa
17821 cagcacactc ttgtatgtc aaccgcgtca atgtggctat cacaaggca aaaattggca
17881 ttgtgtatc aatgtctatg agagatctt atgacaaactt gcaatttaca agtctatgaaa
17941 taccacgtcg caatgtggctt acattacaag cagaaaatgtt aactggactt ttatggact
18001 gtatgtatc caccgcgaa gaagcttcc gtcacgttc tgctgtatc ggctttgtatc
18061 taaatgttca gactgaagga ttatgtgtt acataccagg cataccaaag gacatgaccc
18121 accgtatgtt catctatg atgggttca aatgtatc ccaatgtatc gtttacccatc
18181 atatgtttat caccgcgaa gaagcttcc gtcacgttc tgctgtatc ggctttgtatc
18241 tagaggcttgc tcatgtatc aatgtatc tgggtactaa cttacccatc cagcttagat
18301 ttctacagg tgtaactt gtagtgcatac gactggatc ttgtgtatc gaaaataaca
18361 cagaatttacatc cagatgtatc gcaaaatcc caccaggatc ccaggatc ttatgtatc
18421 cactcatgtatc taaaggcttgc cccatgtatc tagtgcatac taatgtatc cttatgtatc
18481 gtgatgtatc gaaaggatgtatc tccatgtatc tccatgtatc cccatgtatc
18541 agcttacatc aatgtatc ttgtatc tggacatgtatc aatgtatc tttatgtatc
18601 acaaaatgtatc aatgtatc ttgtatc tccatgtatc cccatgtatc aatgtatc
18661 tgggtttgtatc tccatgtatc aatgtatc tccatgtatc tccatgtatc
18721 gtaacccatc gagatgtatc gacatgtatc tggaaatgtatc tccatgtatc
18781 ttgtgtatc tccatgtatc aatgtatc tccatgtatc tccatgtatc tccatgtatc
18841 tgggtttgtatc tccatgtatc aatgtatc tccatgtatc tccatgtatc tccatgtatc
18901 aatgtatc tccatgtatc aatgtatc tccatgtatc tccatgtatc tccatgtatc
18961 acatggaaa tccaaaggcttgc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19021 acatgtatc tccatgtatc aatgtatc tccatgtatc tccatgtatc tccatgtatc
19081 tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19141 ttgtgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19201 tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19261 tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19321 tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19381 tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19441 accgacatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19501 acaaaatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc
19561 atgtgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc tccatgtatc

19621 ttccatcat taataatgtt gttacacaa aggtatgg tattgatgtg gagatcttg
19681 aaaataagac aacacttcct gtaatgtt catttgcgtt tggttcaag cgtaacatta
19741 aaccagtgcc agagattaag atactcaata atttgggtgt tgatatcgct gctaatactg
19801 taatctggga ctacaaaaga gaagccccag cacatgttac tacaatagggt gtctgcacaa
19861 tgactgacat tgccaaagaaa cctactgaga gtgttgttc ttacttact gtctgttig
19921 atggtagagt ggaaggacag gtagacccctt ttagaaacgc ccgttaatgggt gttttataaa
19981 cagaagggtc agtcaaagggt ctaacacccctt caaaggacc agcacaagct agcgtaatg
20041 gagtcacatt aattggagaa ttagttaaaaa cacatgttac tacaataggaa aaagttagacg
20101 gcattattca acagttgcct gaaacctact ttactcagag cagagactta gaggattta
20161 agcccagatc acaaattggaa actgacttc tcgagctcgc tatggatgaa ttcatcacagc
20221 gatataagct cgagggttat gccttcgaac acatgttta tggagatttc agtcatggac
20281 aacttggcggt ttttcatatc atgataggct tagccaagcg ctcacaagat tcaccactt
20341 aatttagagga ttatccctt atggacagca cagtggaaa ttacttcata acagatgcgc
20401.aaacagggtc atcaaaaatgt gtgttgttc tgattgtat ttacttgc gactttgtcg
20461 agataataaa gtcacaagat ttgcgttga ttcaaaaatgtt ggtcaagggtt acaattgtact
20521 atgctgaaat ttcatatc ttttgttga aggatggaca tttttttttt tttttttttt
20581 aactacaagc aagtgcgc tggcaaccagggtt gttttttttt gttttttttt
20641 aaagaatgtc tttttttttt tttttttttt tttttttttt tttttttttt
20701 aaggaataat gatgaatgtc gcaaaatgttcaacttgc tcaataactt aataactt
20761 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
20821 ttgcaccagg tacagctgtt ctcagacaaat gttttttttt tttttttttt tttttttttt
20881 cagatctttaa tgacttcgc tccgcacgtt attctactt aattttttttt tttttttttt
20941 tacatacggc taataatgg gacccatattttttttt tttttttttt tttttttttt
21001 atgtgacaaaa agagaatgtc tttttttttt tttttttttt tttttttttt tttttttttt
21061 agcaaaaactt agccctgggt gttttttttt tttttttttt tttttttttt tttttttttt
21121 ctgacccatattttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21181 atgcacatc atcggaaatgtt tttttttttt tttttttttt tttttttttt tttttttttt
21241 aaattttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21301 atgttgcac tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21361 ctgttgcac tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21421 gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21481 actaaacagaa catgttattttttttt tttttttttt tttttttttt tttttttttt
21541 accgggtgcac cactttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21601 tgagggggggtt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21661 attttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21721 gcaaccctgtt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21781 ttgtccgtgg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21841 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21901 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
21961 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22021 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22081 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22141 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22201 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22261 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22321 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22381 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22441 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22501 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22561 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22621 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22681 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22741 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22801 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22861 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
22921 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt

22981 tagtactttc tttgaacctt ttaaatgcac cgcccacgg tttggacca aaattatcca
23041 ctgacccatt taagaaccag ttgtcaattttaattttaa tggactcaact ggtactggtg
23101 tgtaactcc ttctcaaaag agattcaac cattcaaca attggccgt gatgttctg
23161 atttcactga ttccgttgcga gatcctaaaa catctgaaat attagacatt tcaccitgcg
23221 ctttggggg tgtaagtgtt attacacctg gaacaaatgc ttcatcgaa gttgctgttc
23281 tataatcaaga tgtaactgc actgtatgtt ctacagcaat tcatgcagat caactcacac
23341 cagctggcg catatattct actggaaaca atgtattccca gactcaagea ggctgtctta
23401 taggagctga gcatgtcgac acttcttgcg aatgtcgacat tcctattgga gctggcattt
23461 gtgcttagtta ccatacagtt tcttattac gtgtacttag ccaaaaatctt attgtggctt
23521 atactatgtc tttaggtgt gatagtcaat tgcttactc taataacacc attgctatac
23581 ctactaactt tcaatttgc attactacag aagtaatgcc ttgttctatg gctaaaac
23641 ccgttagattt taatatgtac atctgcggag attctactga atgtgtat ttgcttctcc
23701 aatatggtag ctttgcaca caactaaatc gtgcacttc aggtattgt gctgaacagg
23761 atcgcaacac acgtgaagtq ttgcgtcaag tcaaacaat gtacaaaacc ccaacttga
23821 aataatggg tggtttaat tttcacaaaa tattacctga ccctctaaag ccaactaaga
23881 ggcttttat tgaggacttg ctcttaata aggtgacact cgctgtatgtt ggcttcatga
23941 agcaataatgg cgaatgccta ggtgatatta atgttagaga tctcatttgtt ggcagaatgt
24001 tcaatggact tacagtgtt ccaccttgc tcactgtatgtt gcttacactg
24061 ctgctctatgt tagtggtact gccactgtcg gatggacatt tggctggc gctgcttcc
24121 aaataccctt tgctatgcaaa atggcatata ggttcaatgg cttggatgtt accccaaaatg
24181 ttctctatga gaaccaaaaa caaatgcaca accaattaa caaggcgatt agtcaaaatc
24241 aagaatcaact tacaacaaca tcaactgtatgtt gggcaagct gcaagacgtt gtttaccaga
24301 atgctcaagc attaaacaca cttgttaaac aacttagtgc taattttgtt gcaatttca
24361 gtgtctaaa tgatatccctt tggcgacttg ataaagtgcg ggcggaggta caaaattgaca
24421 ggtaattac aggcaagactt caaaggcttc aaacctatgtt aacacaacaa ctaatcagg
24481 ctgctgaaat caggcttctt gctaatcttgc ctgctactaa aatgtctgatgtt tggcttgc
24541 gacaatcaaa aagagtgtac tttttggaa agggctacca cttatgtcc ttcccacaag
24601 cagccccgca tgggtgttc ttcttacatg tcacgtatgtt gccatcccgg gagagggact
24661 tccaccacagc gccagcaattt tgcgtatgtt gcaaaacataatcccttgcg gaaggtgtt
24721 ttgttttaa tggcacttgc ttgttattt cacagaggaa ctctttctt ccacaaataa
24781 ttactacaga caatacattt gtctcaggaa attgtatgtt cgtttgcg atcattaaaca
24841 acacagttt tgatccctgc caacctgagc ttgactcattt caaagaagag ctggacaatgt
24901 acttcaaaaa tcaatcatca ccagatgtt atcttggcgat catttcaggc attaacgcctt
24961 ctgtcgtaa cattcaaaaa gaaattgacc gcctcaatgtt ggtcgctaaa aattttaatg
25021 aatcactcat tgacccatcaaa gaattggaa aatatgagca atatattttt tggcttgg
25081 atgttggctt cggcttcattt gctgactaa tgccatgtt catggttaca atcttgcattt
25141 gtgtcatgac tagtggtgc agtgcctca agggctatgtt ctcttgcgtt tcttgcgtca
25201 agttgtatgtt ggttgcgttctt gagccatgtt tcaagggtgtt caatttacat tacacataaa
25261 cgaacttgcg gattttttt tgagatttt tactcttgcg tcaatttactg cacagccatgt
25321 aaaaatttgcg aatgttgc tgcgtatgtt tggcgatgtt acagcaacgc taccgttaca
25381 agccctacttc ctttcggat ggcttgcgtt tggcgatgtt ttttgcgtt ttttccag
25441 cgcttccaaa ataatttgcg tcaataaaatg atggcagctt gcccattata agggcttcca
25501 gtccatttgc aatttactgc tgcttgcgtt taccatctat tcaatctt tgcgttgc
25561 tgcgtatgtt gaggcgcaat ttttgcgttctt ctatgcgtt atatattttc tacaatgtatgt
25621 caacgcgtt agaatttata tgagatgtt gcttgcgtt gaaatgtcaat ccaagaaccc
25681 attactttat gatgtcaactt acttgcgtt tggcgatgtt cattttactg actactgtat
25741 accatataac agtgcacag atacaattgtt cttttactg ggtgacggca ttcaacacc
25801 aaaaactcaaa gaagactacc aaatggggg ttattttttt gataggactt caggtgtt
25861 agactatgtc gtgttgcgtt gcttgcgtt cttttttt ttttgcgtt ttttccag
25921 aattactaca gacactggta ttggaaatgc tcaatcttc atctttaaca agcttgc
25981 agacccacccg aatgtcaaa tacacacaat ctttttttgcgtt ttttgcgtt ttttccag
26041 aatggatcca attttatgtt agccgacgac gactactgtc gtgccttgcgtt aagcacaaga
26101 aagtgtatgtt gaaatgtatgtt actcatttgcgtt ttttgcgtt ttttccag
26161 tagcgatgtt ttttgcgtt ttttgcgtt atttttgcgtt ttttgcgtt ttttccag
26221 tgcgtatgtt ttttgcgtt ttttgcgtt ttttgcgtt ttttccag
26281 ggttacgtt ttttgcgtt ttttgcgtt ttttgcgtt ttttccag

26341 ggtctaaacg aactaactat tattattttt ctgttggaa cttaacatt gcttatcatg
26401 gcagacaacg gtactattac cgtttagggag cttaaacaac tcctgaaaca atggAACCTA
26461 gtaatagggtt tccttccct agcctggatt atgttactac aattgccta ttctaatcg
26521 aacaggttt tgtacataat aaagcttggtt tcctctggc tcttggcc agtaacactt
26581 gcttgTTTG tgctgctgc tgtctacaga attaattggg tgactggcgg gattgcgatt
26641 gcaatggctt gtattgttagg ctgtatgtgg cttagctact tcgtgcttc ctccaggctg
26701 ttgctcgta cccgctcaat gtggtcattc aaccagaaaa caaacattct tctcaatgtg
26761 cctctccggg ggacaattgt gaccagaccg ctcatggaaa gtgaacttgt cattgggt
26821 gtgatcattc gtggtcactt gcgaatggcc ggacactccc tagggcgctg tgacattaag
26881 gacctgccaa aagagatcac tgtggctaca tcacgaacgc ttcttatta caaatttagga
26941 gcgtcgccagc gtgttaggcac tgattcaggt ttgctgcat acaaccgcta ccgtatttgg
27001 aactataaat taaatacaga ccacgccggt agcaacgaca atattgctt gctagtacag
27061 taagtgacaa cagatgttc atcttggta ctccaggtt acaatagcag agatattgt
27121 tacattatg aggacttca ggattgctat ttgaaatctt gacgttataa taagttcaat
27181 agtgagacaa ttatTAAGC ctctaactaa gaagaattat tcggagttag atgtgaaga
27241 acctatggag ttagattatc cataaaacga acatgaaaaat tattcttcc ctgacattga
27301 ttgtatTTAC atcttgcag ctatcact atcaggagtg tgtagaggt acgactgtac
27361 tactaaaAGA accttgcCcCA tcaggaacat acgagggcaa ttaccattt caccctctt
27421 ctgacaataa atttgcacta acttgcacta gcacacactt tgctttgct tgtagtgc
27481 gtactcgaca tacctatcag ctgcgtgcaaa gatcagttt accaaaactt ttcatcagac
27541 aagaggaggt tcaacaagag ctctactcgc cacttttctt catttttgc gctctagtt
27601 tttaatact ttgcttcacc attaagagaa agacagaatg aatgagctca cttaatttga
27661 ctctatttgc tgcttttag cccttgcattt attccttggta ttataatgc ttattatatt
27721 ttggTTTCA ctcgaaatcc agatctaga agaacctgt accaaagtct aaacgaacat
27781 gaaacttctc attgtttga ctigtatttc tctatgcagt tgcatatgca ctgttagtaca
27841 ggcgtgtca tctaataaac ctcatgtgct tgaagatcct tgtaggtac aacactaggg
27901 gtaatactta tagcactgct tggctttgt ctttaggaaa ggttttacct tttcatagat
27961 ggcacactat ggttcaaaca tgcacaccta atgttactat caactgtcaa gatccagctg
28021 gtgggtgcgt tatactttagg tgggttacc ttcatgaagg tcaccaact gctgcattt
28081 gagacgtact tggtttta aataaacgaa caaattaaaaa tgctgataa tggaccccaa
28141 tcaaaccAAC gtgtgcccccc ccgcattaca ttgggtggac ccacagattc aactgacaat
28201 aaccagaatg gaggacgcaa tggggcaagg cccaaacagc gccgacccca aggttaccc
28261 aataatactg cgtcttggtt cacagcttcc actcagcatg gcaaggagga acttagattc
28321 cctcgaggcc agggcggtcc aatcaacacc aatagtggtc cagatgacca aattggctac
28381 taccgaagag ctaccgcacg agttcggtt ggtgacggca aatgaaaga gctcagcccc
28441 agatggtact tctattaccc aggaactggc ccagaagctt cacttccctt cggcgctaac
28501 aaagaaggca tcgtatgggt tgcaactgag ggagcccttga atacacccaa agaccacatt
28561 ggcacccgca atcctaataa caatgtgcc accgtgtac aacttcccttca aggaacaaca
28621 ttggccaaaag gcttctacgc agagggaaagc agaggcggca gtcaaggccctt ttctcgctcc
28681 tcatcacgtt gtcgcggtaa ttcaagaaat tcaactccttgc gcagcagtag gggaaattct
28741 cctgctcgaa tggctagcgg aggtgggttacttgccttgc cgctattgct gctagacaga
28801 ttgaaccaggc ttgagagcaa agttcttggtaaaggccaac aacaacaagg cccaaactgtc
28861 actaagaaat ctgtgttca ggcattttttt aagcctcgcc aaaaacgtac tgccacaaaa
28921 cagtacaacg tcactcaacg atttgggaga cgtggccag aacaaacccca agggaaatttcc
28981 ggggaccaag acctaattcag acaaggaact gattacaaac attggccgca aattgcacaa
29041 ttgctccaa gtgcctctgc attcttggta atgtcacgca ttggcatggaa agtcacaccc
29101 tcgggaacat ggctgactta tcatggagcc attaaattgg atgacaaaga tccacaattc
29161 aaagacaacg tcatactgct gaacaagcac attgacgcattt acaaaaacatt cccaccaaca
29221 gagcctaaaaa aggacaaaaa gaaaaagact gatgaagctc agccttgc gcaagagacaa
29281 aagaagcagc ccactgtgac tcttcttcc gccgcgtaca tggatgattt ctccagacaa
29341 ctccaaattt ccatgagtgg agcttctgct gattcaactc aggcataaaac actcatgtat
29401 accacacaacg gcagatggc tatgtaaacg ttccgttacg atacatagtc
29461 tactcttgc cagaatgaat tctcgtaactt aaacagcaca agtaggtttt gttttttt
29521 atctcacata gcaatcttta atcaatgtgtt aacatttaggg aggacttgaa agagccacca
29581 cattttccatc gaggccacgc ggagtacat cgggttaca gtgaataatg cttagggagag
29641 ctgccttatat ggaagagccc taatgtgttta aattttttt agttagtgc tccccatgtg

29701 atttaatag ctcttagga gaatgacaaa aaaaaaaaaaa aaaaaaaaaaa a
//



**Fig. 5. The Subgenomic HCV Replicon Used to Test
The Efficacy of siRNA in Human Liver Cells**



Neo: neomycin phosphotransferase gene

Luc: fruit fly luciferase

EMCV: internal ribosome entry site taken from EMCV

NS3, NS4A, NS4B, NS5A, and NS5B: HCV nonstructural proteins

**Fig. 6. The Effect of siRNAs on HCV Replication
In Huh 5-2 Cells**

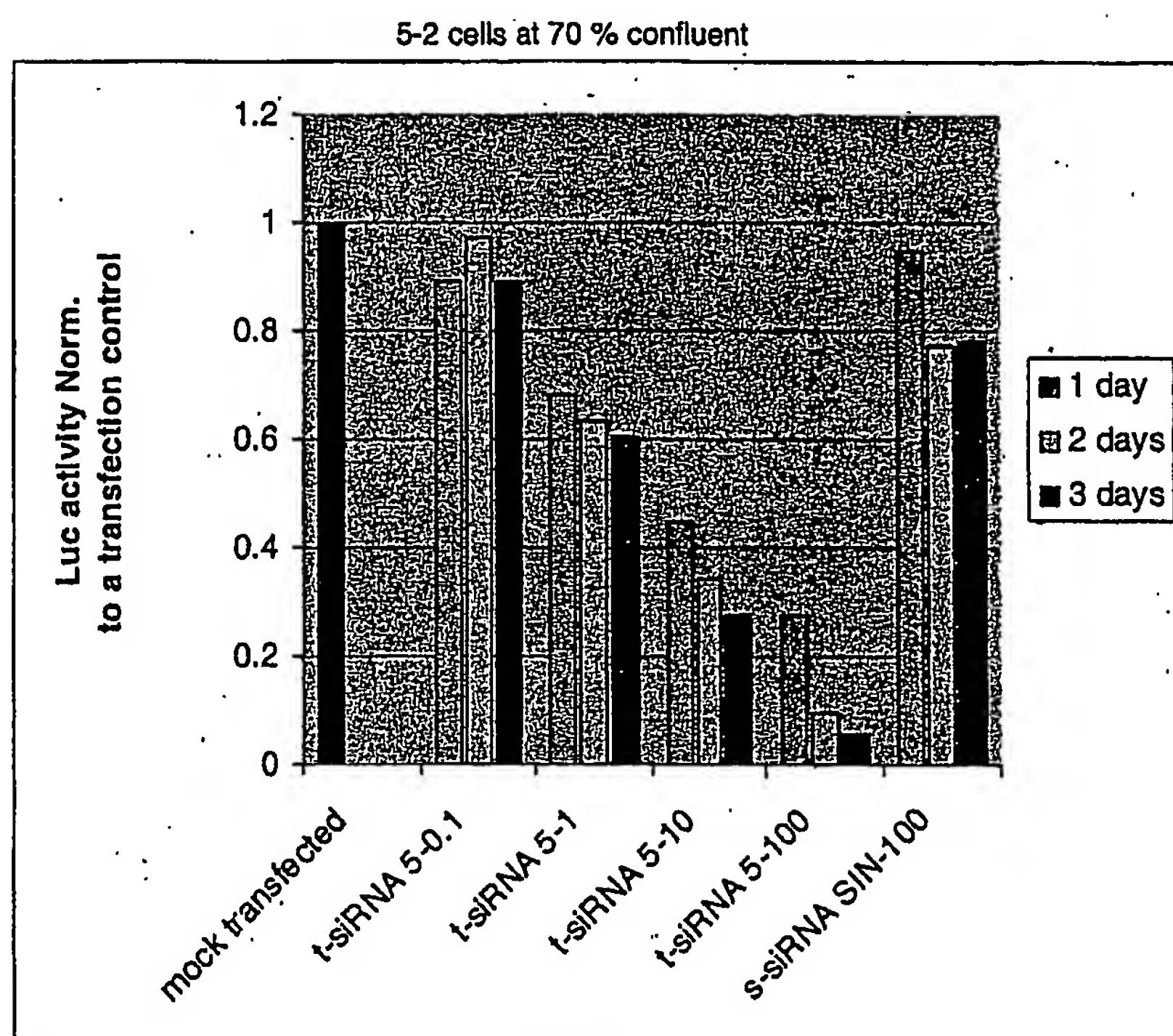


Fig. 7. Sequence Specificity Required for Mediating RNA Interference in Huh7 Cells

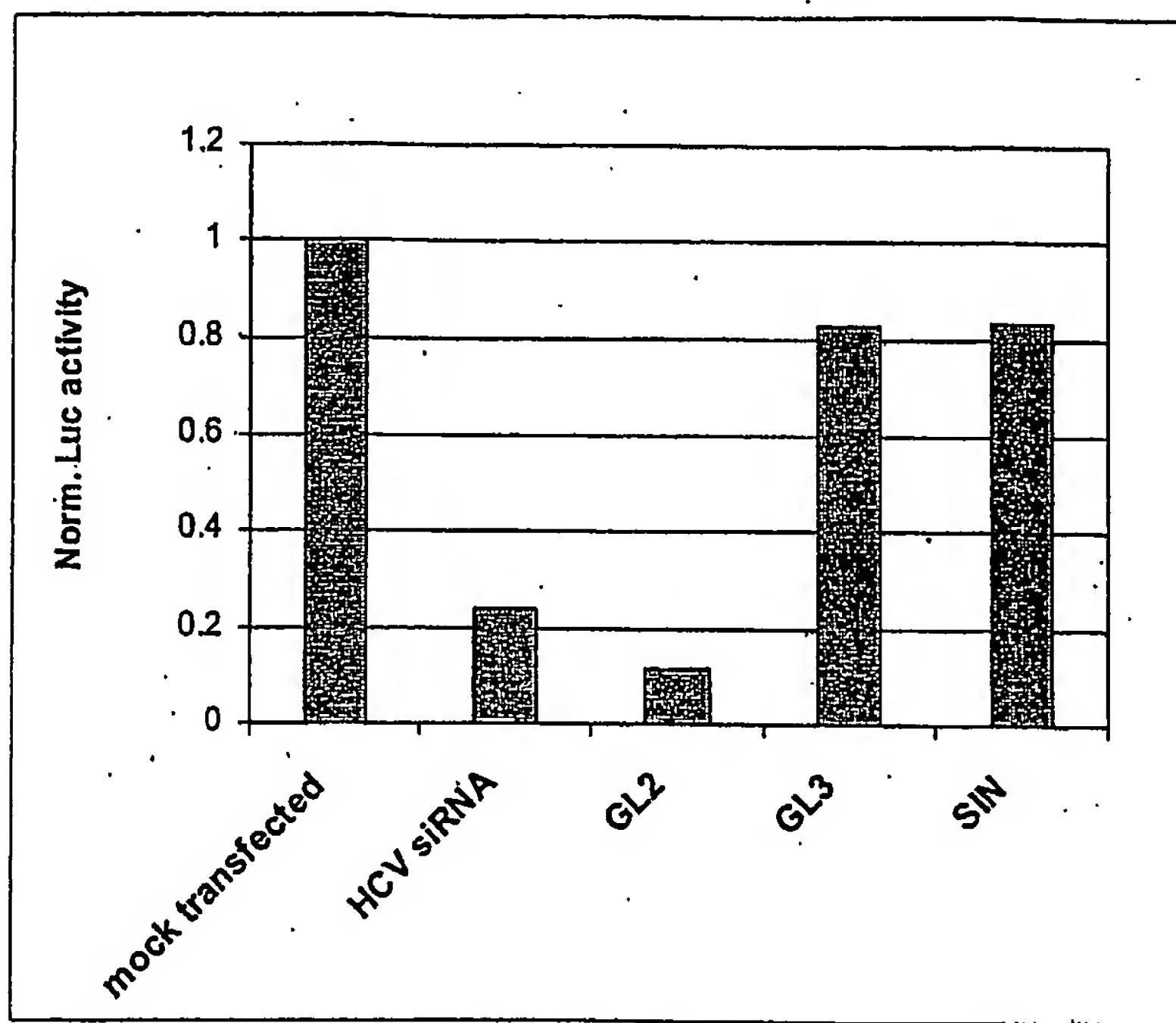


Fig. 8. The Effect of siRNAs of Cell Viability Measured by Cellular ATPase Activity

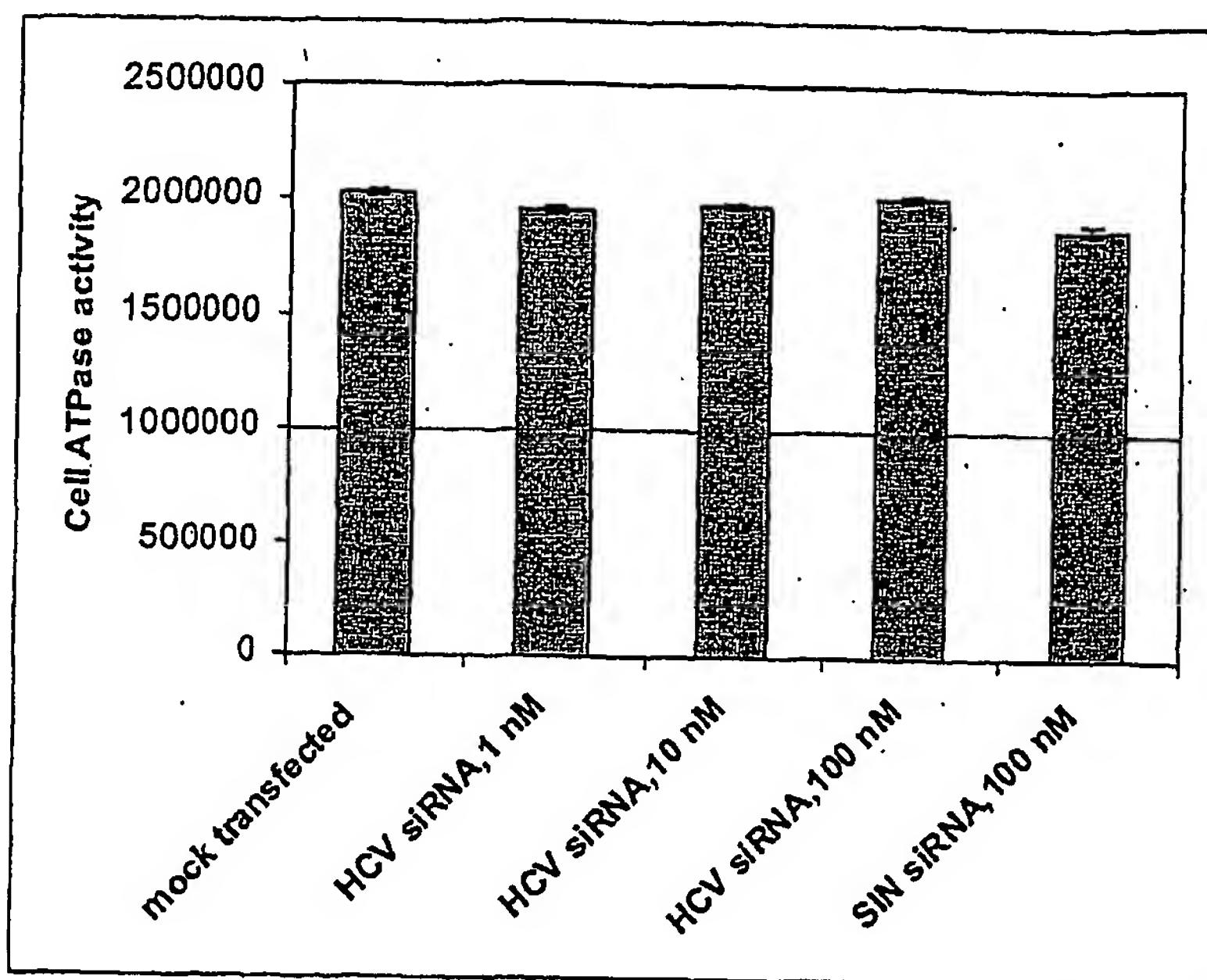


Fig. 9. The Effect of siRNAs₅ on HCV Replication in Huh-7 Cells Measured by HCV RNA Assay

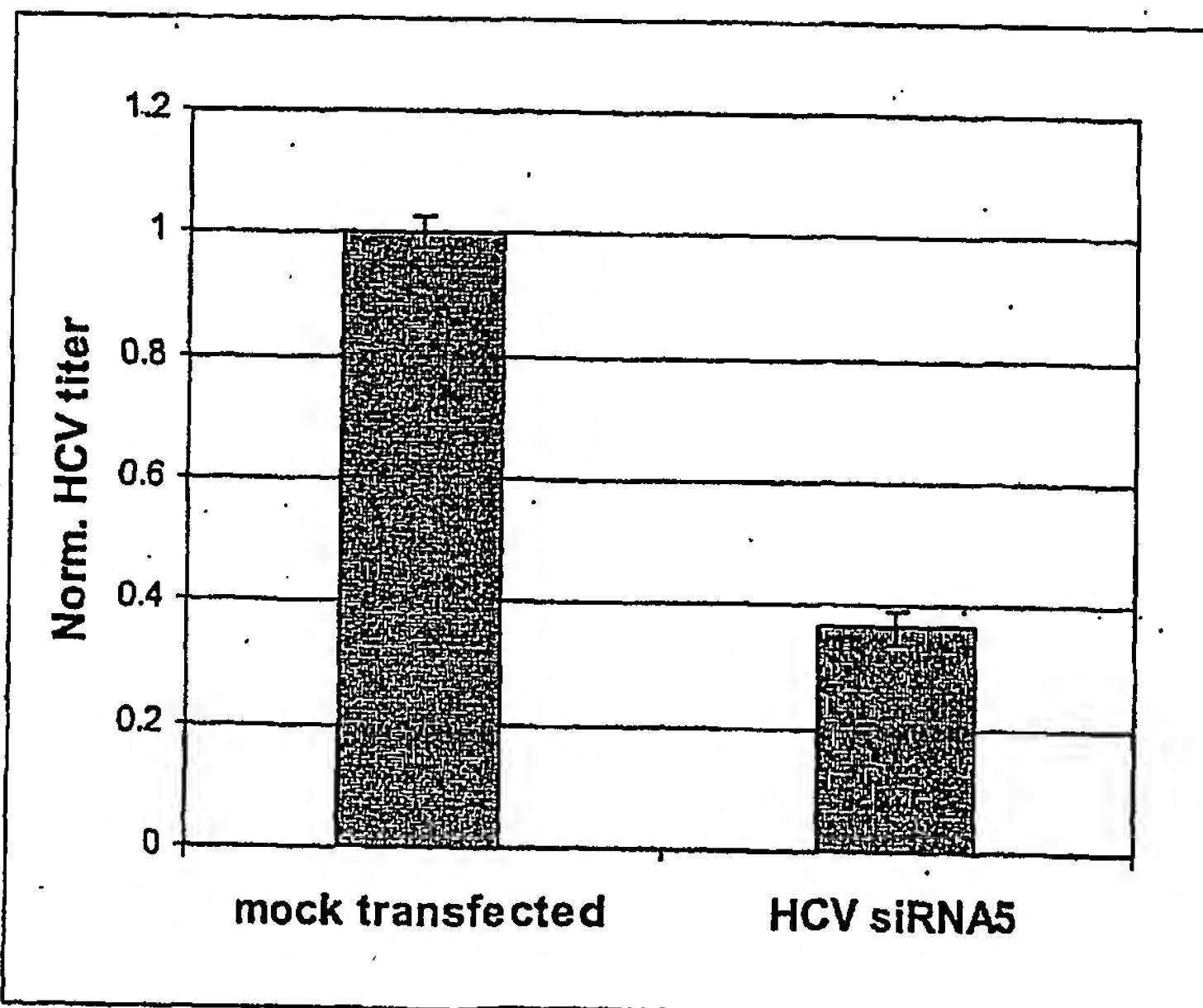


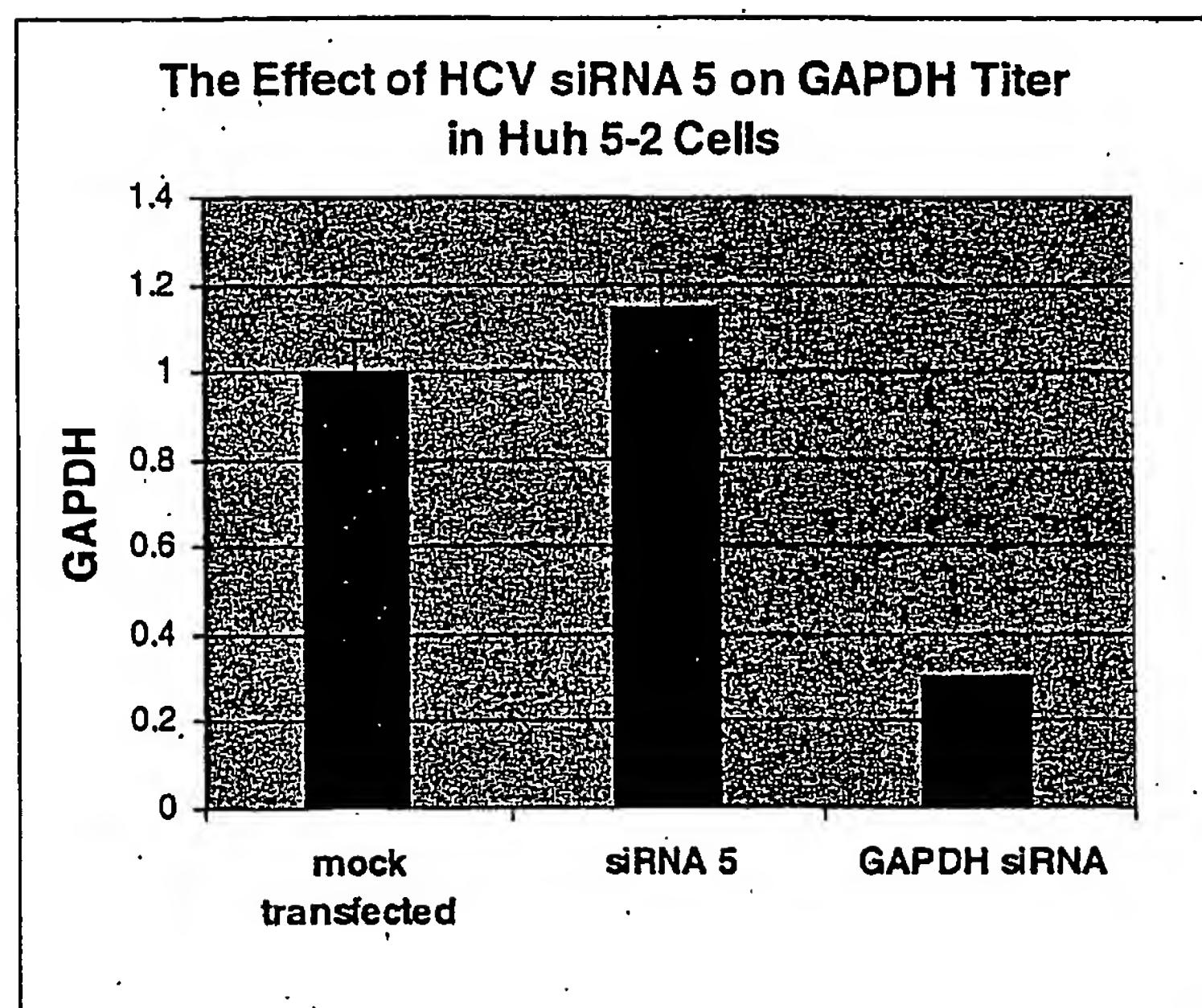
Fig. 10

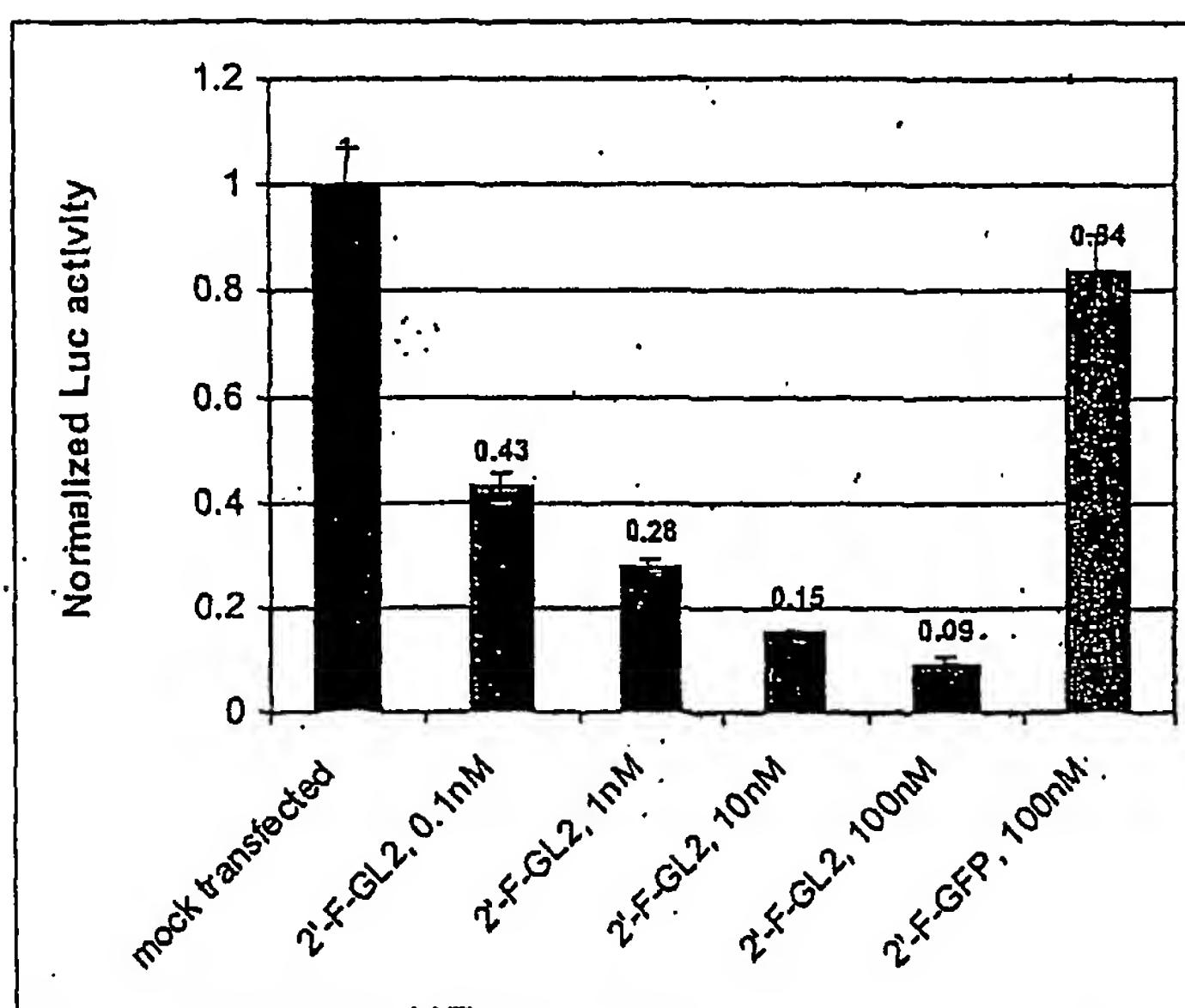
Fig. 11

Fig. 12

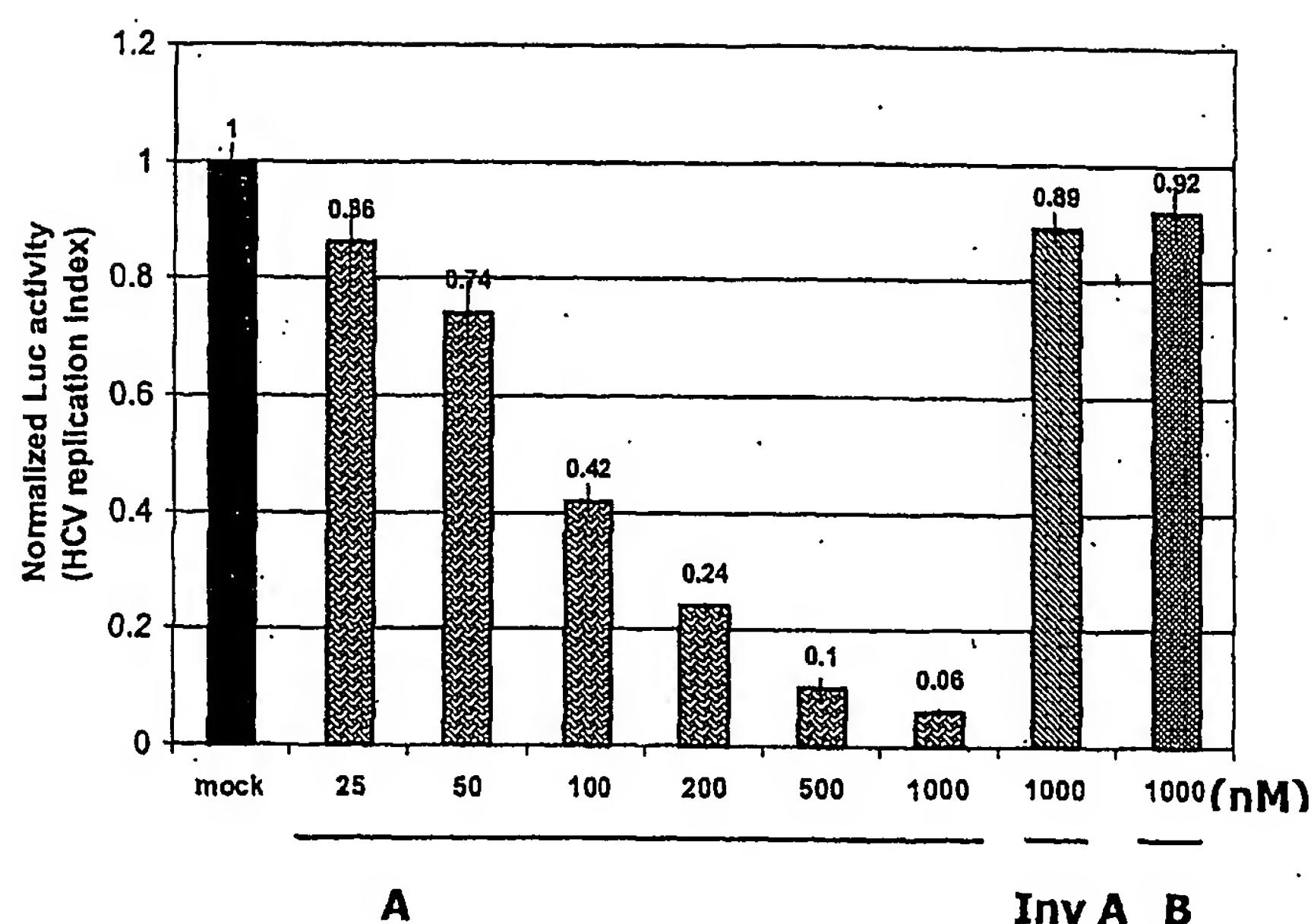


Fig. 13

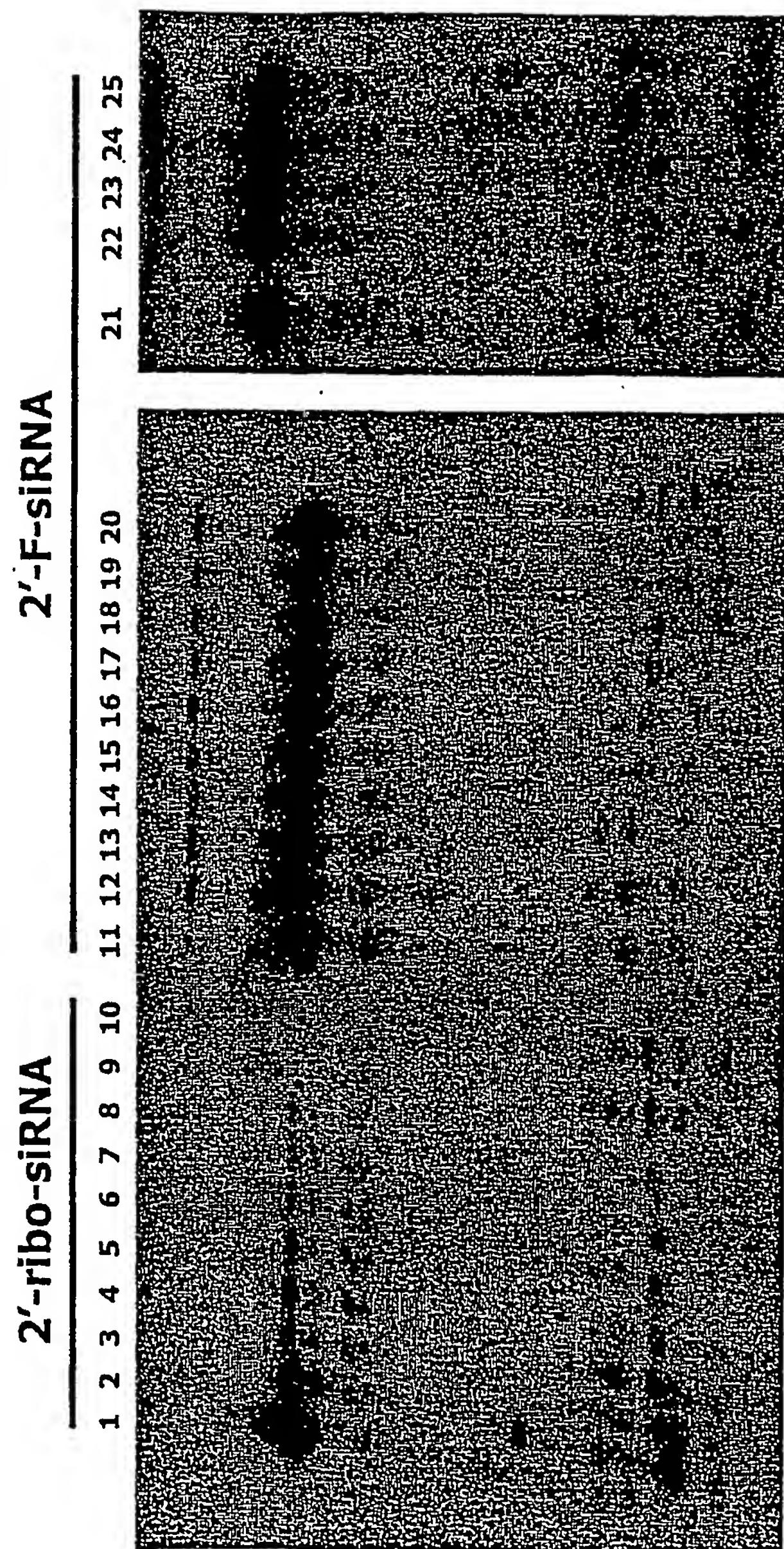
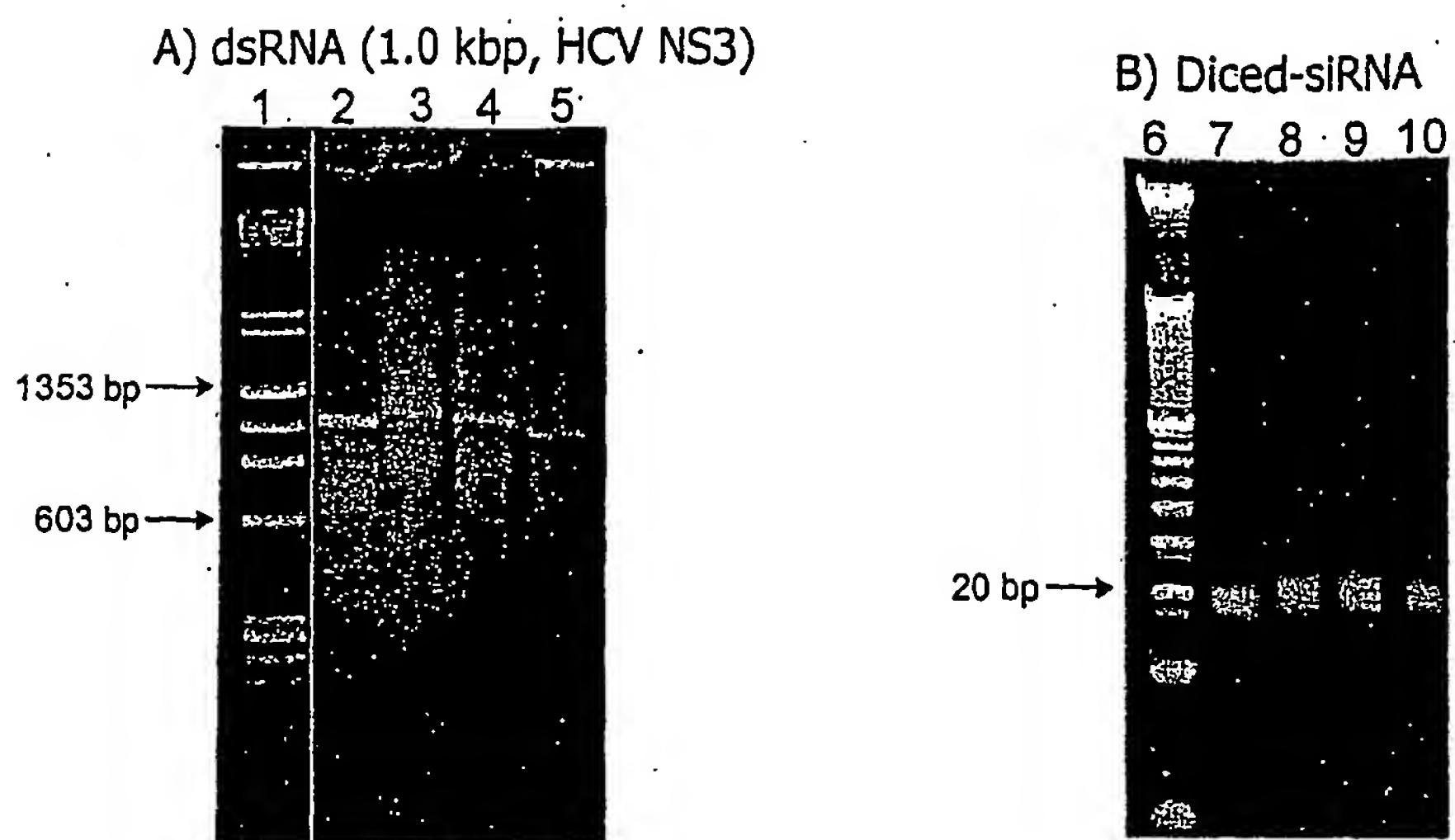


Fig. 14

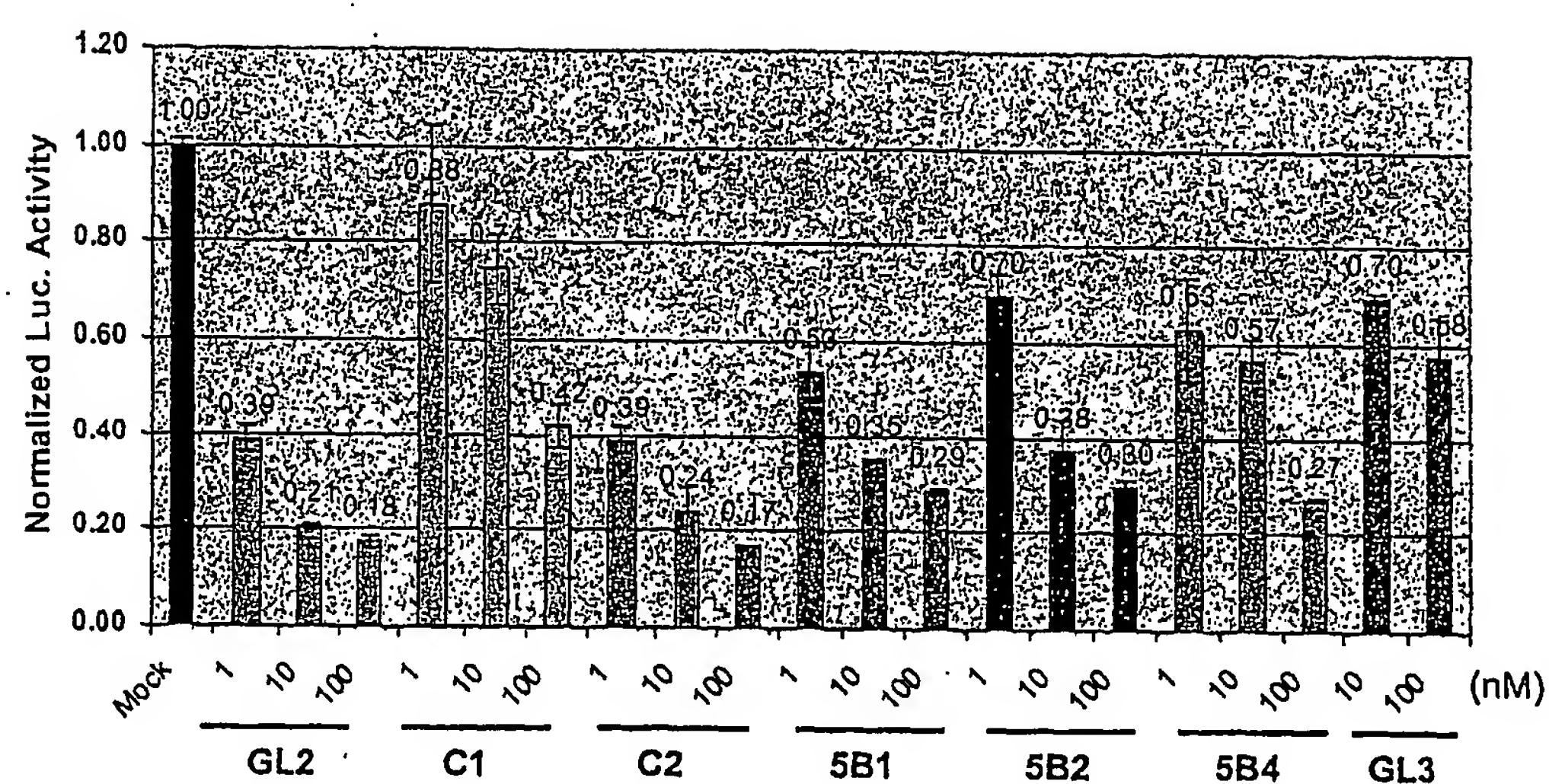


Fig. 15

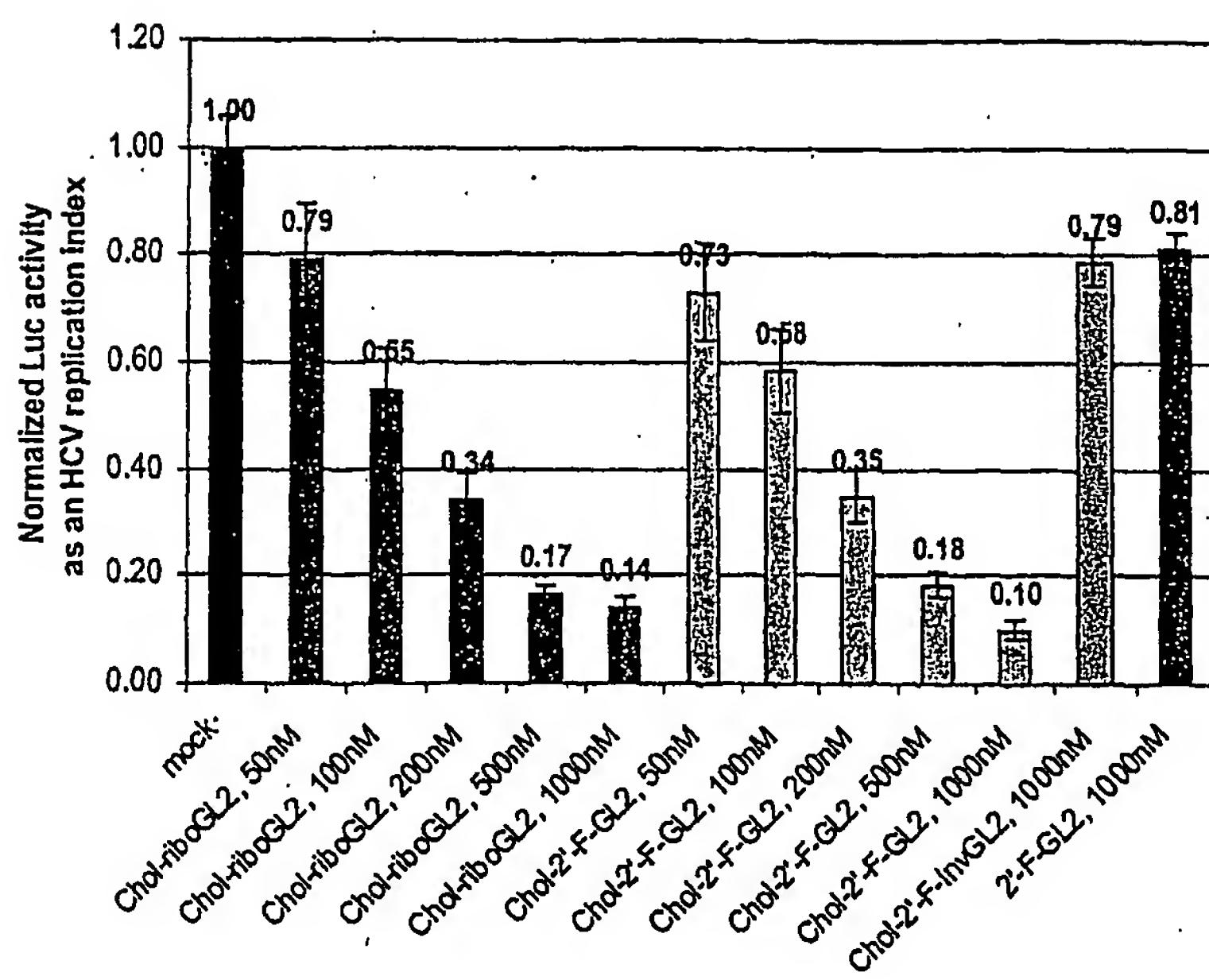


Fig. 16

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.